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Engineering *Escherichia coli* K-12 for the secretion of single domain antibodies against attaching and effacing bacterial pathogens and for the injection of proteins of therapeutic potential into human cells

Doctoral Thesis

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ABBREVIATIONS

A/E	Attaching and effacing
aa	Amino-acid
Ab	Antibody
Ap	Ampicillin
Apram	Apramycin
ARP2/3	Actin-related protein 2/3
AT	Autotransporter
BFP	Bundle forming pili
CDR	Complementarity determining region
C_H	Constant domain of the heavy chain of an immunoglobulin
Cm	Chloramphenicol
CR	*Citrobacter rodentium*
EcN	*E. coli* Nissle 1917
Ecp/Mat	*E. coli* common pilus
EHEC	Enterohemorrhagic *E. coli*
EPEC	Enteropathogenic *E. coli*
FRT	Flippase Recognition Target
Gb3	Globotriaosylceramide
GFP^{TCD}	Green Fluorescent Protein enhanced for the expression in bacteria
GSP	General Secretory Pathway
HA	Hemagglutinin
HCAb	Heavy chain only Ab
H-NS	Histone-like nucleoid-structuring protein
HlyA	α-haemolysin
HR	Homology region
HUS	Hemolytic Uremic Syndrome
IBD	Intimin binding domain
IHF	Integration host factor
IM	Inner membrane
Int	Intimin
Int280	Intimin domain implicated in the Int:Tir interaction
IPTG	Isopropylthio-β-D-galactoside
IRSp53	Homologue of insulin receptor substrate protein of 53 kDa
IRTKS	Insulin receptor tyrosine kinase substrate

K_D	Equilibrium dissociation constant
Km	Kanamycin
LB	Luria-Bertani medium
LEE	Locus of Enterocyte Effacement
MCS	Multicloning site
MOI	Multiplicity of infection
N-WASP	Neural Wiskott–Aldrich Syndrome Protein
Nle	Non-lee effector
o/n	Overnight
OD	Optical density
OM	Outer membrane
PAI	Pathogenicity island
Phab	Phage antibody
RBS	Ribosome binding site
RT	Room temperature
RU	Resonance unit
sdAb	Single domain antibody
SIEC	Synthetic Injector *E. coli*
Sp	Spectinomycin
SPR	Surface plasmon resonance
Stx	Shiga toxin
T1SS	Type I Secretion System
T3SS	Type III Secretion System
Tc	Tetracyclin
TccP	Tir cytoskeleton-coupling protein
Tir	Translocated intimin receptor
TirM	Extracellular domain of Tir
TirC	C-terminal domain of Tir
UPEC	Uropathogenic *E. coli*
V_H	Variable domain of the heavy chain of an Ig
V_{HH}	Variable domain of the heavy chain of a HCAb, also called *nanobody*
V_L	Variable domain of the light chain of an Ig

SUMMARY

The attaching and effacing (A/E) bacterial pathogens infect the gastrointestinal tract of humans and other mammals after ingestion of contaminated food or water and cause persistent diarrhoea and other important diseases (e.g. haemolytic uremic syndrome, HUS) worldwide. Prototypical A/E pathogens are the enteropathogenic *Escherichia coli* (EPEC) and enterohaemorrhagic *E. coli* (EHEC) strains, which infect humans, as well as the mouse-restricted pathogen *Citrobacter rodentium* (CR). These pathogens contain a common type III secretion system (T3SS): a macromolecular protein complex (the injectisome) assembled in the bacterial cell envelope that protrudes to the extracellular milieu with a filament of polymerized EspA. The T3SS allows translocation (injection) of a repertoire of bacterial proteins (called effectors) into the cytoplasm of the host enterocytes through the translocon subunits EspB and EspD, which insert in the host cell membrane. The effectors subvert multiple cellular functions and cause the effacement of the intestinal microvilli (A/E lesions) and the disruption of the intestinal epithelial barrier. Among them, the translocated intimin receptor (Tir) inserts in the host cell plasma membrane and is recognised by Intimin (Int), an outer membrane adhesin exposed on the bacterial cell surface. The Int:Tir interaction promotes the intimate attachment of the bacterium to the enterocyte and the polymerization of actin filament bundles (called "pedestals") underneath the attached bacterium. Effective treatments to combat A/E pathogens are needed, since antibiotics activate the expression of the life-threatening Shiga-like toxins (Stx) from integrated pro-phages, which are present in the more virulent strains - such as EHEC O157:H7. In this work we have assessed whether single domain antibodies (sdAbs) from camelids (also known as nanobodies or V_{HH}s) binding essential proteins for A/E lesion formation (i.e., EspA, Int, Tir) could act as potential therapeutic agents against EHEC O157:H7 infections if secreted from non-pathogenic *E. coli* strains. In addition, we have explored the biotechnological use of the filamentous T3SS of the A/E pathogens to inject V_{HH}s and other proteins with therapeutic potential into the cytoplasm of human cells using a non-pathogenic *E. coli* K-12 strain, engineered to express functional EPEC injectisomes.

We have immunized a dromedary with purified EspA, Int280 and TirM (the protein domains involved in Int:Tir interaction) of EHEC to create a library of V_{HH} genes. Selected V_{HH}s clones from this library were secreted by commensal *E. coli* K-12 strains carrying the α-haemolysin (HlyA) secretion system and purified from the extracellular medium to characterise their binding and inhibitory properties of Int:Tir interaction *in vitro*. A high-affinity V_{HH} clone recognising TirM - named TD4 - and blocking Int:Tir interaction was found to interfere with the formation of actin pedestals on HeLa cells infected with EHEC. We have demonstrated that TD4 effectively competes with Int280 for the binding of Tir, since it has higher affinity towards TirM and recognises an epitope that overlaps with the necessary residues for the Int:Tir interaction. This V_{HH} showed high specificity towards TirM of EHEC, not binding TirM of EPEC and only weakly with TirM of *C. rodentium.* With the aim to establish an *in vivo* mouse model for the evaluation of the A/E inhibition by

TD4, we generated a *C. rodentium* strain expressing EHEC proteins Int, Tir, the multicargo chaperone CesT and the Tir-coupling protein effector TccP. The resulting strain (CR-EHEC) was capable of forming actin pedestals on HeLa cells, colonizing the mouse intestinal tract and inducing crypt hyperplasia, similarly to wild type *C. rodentium*. Importantly, we confirmed that TD4 also inhibits the formation of actin pedestals on HeLa cells induced by CR-EHEC. These results leave open the possibility of testing the activity of TD4 against the formation of A/E lesions *in vivo*, which could represent the basis of a future EHEC infection treatment to control the outbreaks of this pathogen.

In addition, we have addressed the biotechnological use of the filamentous T3SS of A/E pathogens for the injection of sdAbs and other heterologous proteins of therapeutic potential into the cytoplasm of human cells. The genes encoding the T3SS of EPEC are found in a 35 kb chromosomal island, called the Locus of Enterocyte Effacement (LEE). LEE is organized in various large operons (LEE1 to LEE5) and shorter transcriptional units (e.g. escD) and also contains genes encoding some effectors, their chaperones, transcriptional regulators, a muramidase and intimin. We aimed to engineer the expression of all known necessary genes for the assembly of a functional T3SS (27 genes in EPEC) in a non-pathogenic *E. coli* strain (e.g. K-12) under the control of the inducible promoter Ptac. We organised these genes in five engineered transcriptional units - operons eLEE1 to eLEE4 and eEscD - lacking effectors and transcriptional regulators. These engineered operons were sequentially integrated in specific sites of the genome of *E. coli* K-12 corresponding with fimbrial and afimbrial adhesins. The resulting non-pathogenic strain, referred to as Synthetic Injector *E. coli* (SIEC), was demonstrated to express the T3SS genes upon IPTG addition, to assemble functional injectisomes on its envelope and to be able to secrete the translocator proteins EspA, EspB, EspD. Interestingly, we found that the expression of the T3SS genes partially interfered with the assembly of the flagellum in *E. coli* K-12, which can be explained considering the high identity between components of the T3SS and the flagellum. Lastly, we showed that a SIEC strain carrying an additional engineered operon encoding Int, Tir and CesT (eLEE5), was able to inject Tir to the cytoplasm of HeLa cells and to reproduce the intimate attachment of the bacterium to the host cell and the formation of actin pedestals. These results open the possible biomedical application of SIEC-derived strains to inject proteins of therapeutic potential (e.g. V_{HH}s, enzymes, transcription factors, toxins, effectors) against diverse human diseases (e.g. cancer, chronic inflammation).

PRESENTACIÓN

Las bacterias patógenas que producen lesiones de unión y borrado (A/E) de los microvilli intestinales colonizan el tracto gastrointestinal de humanos y otros mamíferos cuando estos ingieren comida o agua contaminados. Estas bacterias causan diarreas severas y otras enfermedades (como el síndrome urémico hemolítico, HUS) en todo el mundo. Los patógenos A/E prototípicos son las cepas de *Escherichia coli* enteropatogenas (EPEC) y enterohemorrágicas (EHEC) que infectan humanos, y el patógeno murino *Citrobacter rodentium*. Todos ellos codifican un sistema de secreción tipo III (T3SS), un complejo proteico también denominado inyectisoma que se ancla en la envuelta celular de estas bacterias y extiende al medio extracelular un filamento formado por la polimerizacion de la proteína EspA. El T3SS permite la translocación (inyección) de una serie de proteínas bacterianas (denominadas efectores) al citoplasma de los enterocitos infectados a través de un traslocón formado por las proteínas EspB y EspD, que forman un poro en la membrana plasmática del enterocito. Los efectores interfieren con numerosas funciones celulares, promueven la desaparición (borrado) de los microvilli intestinales (lesiones A/E) y rompen la hoemostasis del epitelio intestinal. Entre los efectores, el receptor translocado de intimina (Tir) se inserta en la membrana plasmática de la célula infectada y es reconocido por intimina (Int), una adhesina expuesta en la membrana externa de la bacteria. La interacción Int:Tir desencadena una unión íntima de la bacteria al enterocito y la polimerización de filamentos de actina bajo la zona de unión de la bacteria, formando una estrucutra denominada "pedestal de actina". Todavía no disponemos de tratamientos eficaces para combatir los patógenos A/E, dado que los antibióticos activan la expresión de las peligrosas toxinas Shiga (Stx) desde pro-fagos integrados en el genoma de las cepas más virulentas, como EHEC O157:H7. En este trabajo, evaluamos la posibilidad de usar cepas no patógenas de *E. coli* para secretar anticuerpos monodominio (sdAbs) derivados de camellos, también conocidos como nanobodies o V_{HH}s, que se unan a proteínas esenciales para la formación de las lesiones A/E (como son EspA, Int y Tir) y que actúen como posibles agentes terapeúticos contra las infecciones de EHEC O157:H7. Además, hemos testado el posible uso biotecnológico del T3SS de los patógenos A/E como método de inyección de V_{HH}s y otras proteínas con potencialidad terapéutica al citoplasma de células humanas usando una cepa no patógena de *E. coli* (K-12) modificada para expresar inyectisomas de EPEC.

Hemos inmunizado un dromedario con las proteínas EspA, Int280 y TirM (los dominios proteicos implicados en la interacción Int:Tir) purificadas para crear una genoteca de V_{HH}s. Los clones que seleccionamos por su capacidad de unión a estos antígenos fueron secretados por una cepa comensal *E. coli* K-12 que expresa además el sistema de secreción de la α-hemolisina (HlyA). Los V_{HH}s se purificaron del medio extracelular para caracterizar sus propiedades de unión y de inhibición de la interacción Int:Tir *in vitro*. Demostramos que un clon de alta afinidad contra TirM - denominado TD4 - es capaz de bloquear la interacción Int:Tir y evitar la formación de pedestales de actina en cultivos de células HeLa infectadas con EHEC. Mostramos

además que TD4 compite con Int280 por la unión de Tir, dado que posee una mayor afinidad por esta proteína y que reconoce un epítopo de TirM que solapa con el sitio de unión de Int280. Este V_{HH} mostró alta especificidad hacia TirM de EHEC, puesto que no se unió a TirM de EPEC y lo hizo sólo con baja afinidad a TirM de *C. rodentium*. Para poder establecer un modelo animal necesario para evaluar el efecto protector de TD4 de prevención de la formación de las lesiones A/E *in vivo*, modificamos *C. rodentium* para expresar las proteínas de EHEC Int, Tir, la chaperona CesT y el efector TccP (la proteína de acoplamiento de Tir_{EHEC}). La cepa resultante (CR-EHEC) fue capaz de formar pedestales de actina sobre células HeLa en cultivo, de colonizar el tracto intestinal de ratones y de inducir hiperplasia en la criptas intestinales, al igual que lo hace la cepa silvestre de *C. rodentium*. Además, confirmamos que TD4 también inhibe la formación de pedestales de actina en células HeLa infectadas con CR-EHEC. Estos resultados abren la posibilidad de comprobar el efecto de TD4 *in vivo*, lo que podría derivar en un tratamiento más eficaz contra EHEC para controlar los brotes de este patógeno.

Por otro lado, evaluamos el posible uso biotecnológico del T3SS filamentoso de los patógenos A/E para la inyección de sdAbs y otras proteínas heterólogas con potencial terapéutico al citoplasma de células humanas. Los genes que codifican el T3SS de EPEC se encuentran en una isla cromosómica de 35 kb denominada LEE (*locus of enterocyte effacement*) que está organizada en varios operones grandes (LEE1 a LEE5) y otras unidades transcripcionales monocistrónicas (como *escD*). LEE también contiene genes que codifican seis efectores y sus chaperonas, varios reguladores transcripcionales, una muramidasa e intimina. Diseñamos mediante la ingenirería genética la expresión de todos aquellos genes necesarios para el ensamblaje del T3SS funcional (27 genes en EPEC) en una cepa no patogénica de *E. coli* (K-12) bajo el control del promotor inducible Ptac. Organizamos estos genes en cinco unidades transcripcionales artificiales (operones eLEE1 a eLEE4 y eEscD) que carecían de secuencias codificantes de efectores y de reguladores transcripcionales, y los integramos secuencialmente en sitios específicos del cromosoma de *E. coli* K-12 que codifican adhesinas fimbriales y afimbriales. La cepa resultante, no patógena, se denominó *E. coli* inyectora sintética (SIEC) y demostramos su capacidad para expresar los genes del T3SS trás la inducción con IPTG, de ensamblar inyectisomas funcionales en sus membranas y de secretar las proteínas translocadoras EspA, EspB y EspD al medio. Además, vimos que la expresión de los genes del T3SS interfieren parcialmente con el ensamblaje del flagelo en *E. coli* K-12, un hecho que se explica dada alta homología que comparten los componentes del T3SS y el flagelo. Finalmente, mostramos que SIEC, modificada para expresar Int, Tir y la chaperona CesT (eLEE5), es capaz de inyectar Tir al citoplasma de células HeLa y de reproducir la unión íntima de la bacteria a la célula infectada formando pedestales de actina. Estos resultados abren la posibilidad al desarrollo de aplicaciones biomédicas con cepas derivadas de SIEC inyectando proteínas con potencial terapéutico (como V_{HH}s, enzimas, factores de transcripción, toxinas o efectores) para tratar distintas enfermedades (p. ej. cáncer o la inflamación crónica intestinal).

INTRODUCTION

Intestinal microbiota and disease

Among microorganisms, bacteria have adapted to almost every possible habitat on the planet, including salt deserts, the bottom of oceanic trenches or even power plants[48]. But their greatest adaptation success in terms of number has been achieved in association with other organisms, from protists[185] to humans[361]. Microbial communities established these associations far before the appearance of mammals[81] forming a landscape of bacterial interactions called microbiota. In terrestrial vertebrates they are found on the skin and mucosal surfaces, but the intestinal lumen hosts a specially large, complex and dynamic collection of microorganisms[142]. In certain parts of the intestine, bacteria are particularly high concentrated, reaching up to 10^{12} bacteria per g (dry weight) in the human colon, which makes adults numerically more prokaryotic than human, since it has been estimated that only 10% of our cells are eukaryotic[315]. However, humans are essentially born germ-free. After birth, up to 500 independent species or phenotypes from 30 different genera colonize the intestine. High-throughput sequencing has recently allowed complex microbial communities to be characterized, even microorganisms that cannot be easily cultured[88]. These approaches have identified that the genus *Bacteroides* is the most common among the obligate anaerobes, while it is *Escherichia* among the facultative anaerobes[343]. The bacterial lineages present in humans are related to those in other terrestrial vertebrates, indicating that humans have co-evolved with their microbial partners[81]. This co-evolution can be explained from the fact that bacteria and the host establish interspecific relationships that contribute to the benefit of both of them: commensalism (one partner benefits and the other is unharmed) and mutualism (both partners benefit).

Recent reports have demonstrated that the balanced interplay between the intestine and the intestinal microbiota affects to multiple aspects of the mammalian physiology: it plays a determining role in the maturation of a fully functional immune system[161], the mucosal cell turnover and the vascularity of the intestine[219]. Germfree animal models have also shown that the intestinal microbiota can be considered as an additional organ that regulates the general host homeostasis[394]. More importantly, otherwise inaccessible nutrients for the host are produced as bacterial metabolic by-products[23,143]. As an example, polysaccharides ingested from plants are not digestible, but are main substrates for colonic bacteria, while the products of microbial fermentation constitute important energy sources for the host[103]. In these terms, the host is relieved of the need to evolve a wider metabolic adaptability[142]. The more access get the bacteria to a nutrient-rich niche protected from the environment where they can multiply, the more benefit gets the host from the by-products of the bacterial metabolic activity[304]. The mutual and beneficial relationship is dynamic and also serves as a protective barrier against the colonization of the intestinal epithelium by external bacteria, including potential pathogens; bacteria compete for the nutrient sources and maintain the collective microenvironment[104,228]. However, different external cues such as stress, antibiotic treatments or changes in the diet may unsettle the intestinal microbiota[49,56]. This situation is

termed dysbiosis and - if maintained - can act as a starting point in the infection process of opportunistic or specialised pathogens. The latter make use of acquired virulence factors to take advantage of exposed suitable niches[324] upon infection and perturb cell homeostasis, using strategies that range between internalisation from the apical surface, disruption of the cell junctions and cytotoxic injury[49]. To avoid the immune response, specialised pathogens also suppress specific signal cascades that lead to the inflammation of the epithelium[374].

Attaching and effacing pathogens

An interesting member of the human microbiota is commensal *Escherichia coli*. It resides in a highly specific niche of the colon, where it succesfully competes with other species for the available gluconate[342]. However, it can cause infection as an opportunistic pathogen when the gastrointestinal barriers are disrupted. Besides, several high adapted strains of *E. coli* have acquired pathogenicity islands (PAI), prophages and plasmids that help them to circumvent the immune system, compete with other bacteria and efficiently colonize specific anatomical sites[66]. They cause disease to either animals and humans worldwide[164,408] and infections may be limited to the mucosal surfaces or can disseminate throughout the body. Though some strains cause extra-intestinal infections, e.g. in the urinary tract (uropathogenic *E. coli*, UPEC)[66,164,224], they are mainly diarrhoeagenic strains[58,251]. Among them, the denominated attaching and effacing (A/E) pathogens[107] are of particular interest for this work. The human pathogens enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) belong to this group[66,251,297], as well as the mouse-restricted pathogen *Citrobacter rodentium* (CR)[59].

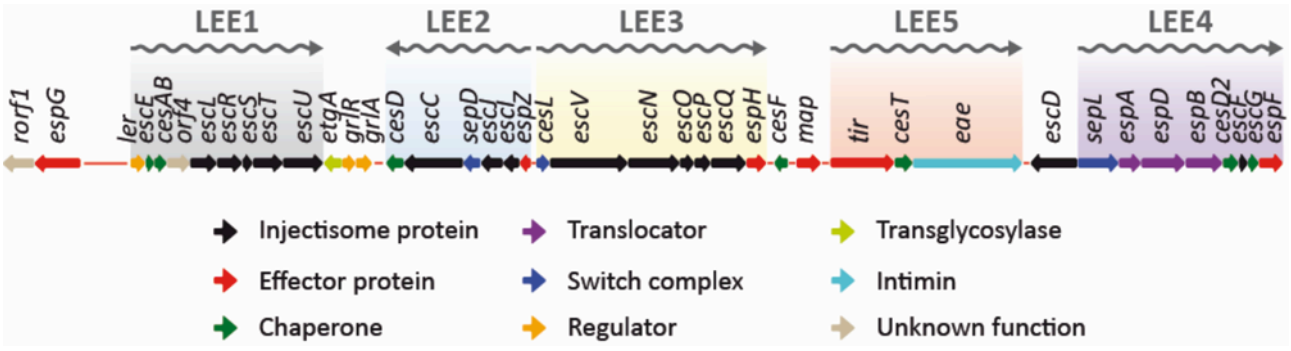


Figure 1. The Locus of Enterocyte Effacement (LEE). This genomic island is organised in five main operons and other minor transcriptional units. It harbours all the necessary genes to assemble a functional T3SS and other proteins related to it, including regulators and some of the effector proteins. The scheme corresponds exactly with the LEE of EPEC. Minor differences are found in the LEE of EHEC and CR.

The A/E pathogens have in common a ca. 35 Kb PAI, termed Locus on Enterocyte Effacement (LEE)[233,316]. The G+C content of the LEE is about a 38%, which is much lower than the 50% of the total *E. coli* genome[233], thus suggesting horizontal transfer of this pathogenicity island into *E. coli* from another species. In spite of differences in size and genetic content between A/E pathogens, LEE PAI maintains a basic structure that

includes 41 genes organized in main 5 polycistronic operons: LEE1, LEE2, LEE3, LEE4, LEE5; two bicistronic operons and 4 monocistronic entities[398] (Figure 1). These transcriptional units are directly related with the expression of a Type III Secretion System (T3SS) on the bacterial cell envelope[78,92], which is a needle-like macromolecular complex necessary for the capacity of the A/E pathogens to inject proteins to the host cells[92]. The LEE also encodes chaperones, regulators, a lytic transglycosilase[112] and effector proteins, including the main virulence factors for the pathogenesis: the outer membrane (OM) adhesin intimin and its receptor, Tir. To cause infection, the pathogens colonize the intestinal mucosa and bind to the apical surface of host enterocytes. In the infection site, they inject virulence factors through the T3SS[377] to manipulate the actin cytoskeleton and form distinct pedestals structures beneath the site of attachment[177] that lead to the effacement of the brush border microvilli[107]. These histopathological lesions - termed A/E lesions - can be observed in intestinal biopsies from patients or infected animals and can be reproduced *in vitro*. The A/E pathogens share this common strategy for infecting (Figure 2), which can be summarized in three main stages: i) localized adherence, ii) intimate attachment and signal transduction, and iii) alteration of cell homeostasis.

i) Localized adherence. Infection begins with ingestion of contaminated food or water. Acid resistance[258] facilitates the survival of the bacteria through the low pH of the stomach and environmental signals of the intestine turn on their virulence factors[131,358]. These signals include temperature[363], hormones adrenaline and noradrenaline or the quorum-sensing molecule auto-inducer 3 produced by the gastrointestinal cells[144,302,335]. The pathogens integrate these signals with internal specific and global transcriptional regulators: PerC (EPEC)/PchABC (EHEC), the histone-like nucleoid-structuring protein (H-NS)[40] and the integration host factor (IHF), among others[231]. The coordinate effects of the signals activate the transcription of the LEE and of distinct fimbrial and non-fimbrial adhesins that participate in the initial attachment to enterocytes. In addition, flagella, the EspA filament of the T3SS[108] and the *E. coli* common pilus (Ecp/Mat)[307] are thought to be involved in the adhesion of the A/E pathogens, although the different pathotypes may possess specific means to adhere to the host cells (commented below).

The expression of the LEE is highly controlled. Apart from the external cues, the physiological state of the cell (e.g. the growth phase) and regulators encoded in the own LEE determine the expression of the T3SS[358]. The first gene encoded in operon LEE1 - *ler* - is the master transcriptional regulator of the LEE PAI[28]. Constitutively expressed at low levels, Ler activates the transcription of operons LEE2-LEE5 counteracting the inhibitory effect of H-NS[40,388], but also functions as a repressor of LEE1[22,25], creating a negative feedback loop. Extra-LEE genes that are known to be regulated by Ler in EPEC include *espC*[237], encoding an autotransporter extracellular serine protease, that is thought to play various roles in pathogenicity[253,309]. In contrast, the EHEC homologue of *espC* (*espP*) is not Ler-regulated[91]. Besides Ler, other regulator proteins of

the T3SS expression are GrlR (that acts like a negative regulator) and GrlA (a positive regulator)[159,302], which are organized together in a single operon of the LEE[13].

ii) Intimate attachment and signal transduction. After the first contact with the host cells, the A/E pathogens assemble protein translocation pores in the host plasma membrane formed by EspB and EspD and inject their own receptor through the T3SS to the cytoplasm, which is a 78 kDa protein referred to as translocated intimin receptor (Tir). Tir is directly phosphorilated by serine/threonine kinases in its C-terminal tail (TirC)[194,252] to be inserted into the apical cell membrane of the enterocytes[171]. It leaves an extracellular receptor domain of about 100 residues (TirM) - composed of two helices separated by a loop[169] - to be accessible for the recognition of the C-terminal lectin-like domain of the 95 kDa intimin adhesin[171]. N- and C-terminal portions of Tir are cytosolic. The N-terminal tail of Tir is, as TirM, highly conserved among the A/E pathogens, and binds various focal adhesion proteins linking Tir to the cytoskeleton[123]. The more divergent TirC domain - EPEC and EHEC only share 41% homology - contains tyrosine residues that are subject to phosphorylation.

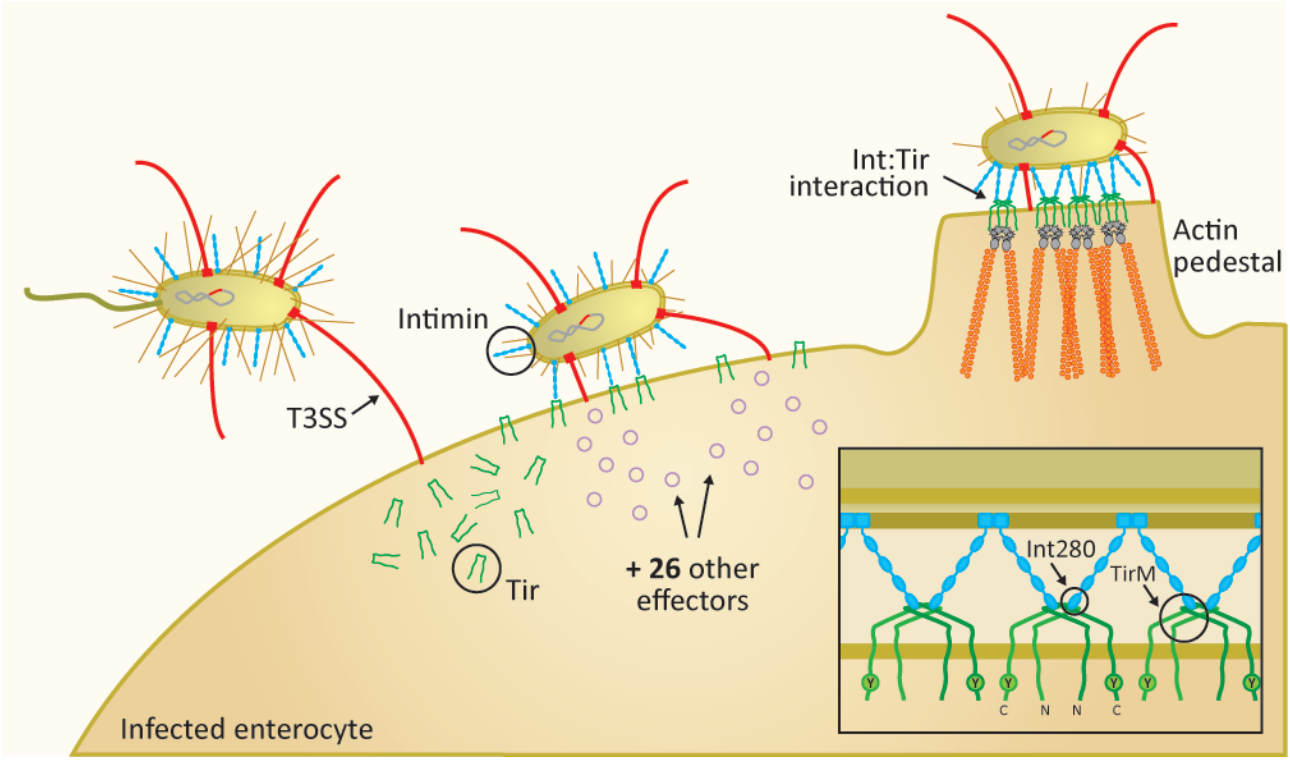


Figure 2. Infection process of the A/E pathogens. After a first contact with the host cell, the pathogen injects Tir and other effectors (at least 27 in EPEC, 62 in EHEC and 35 in CR)[391] to the cytoplasm through the T3SS. Tir exposes on the membrane of the eukaryotic cell its central domain (TirM) and is recognised by the C-terminal lectin-like domain of intimin. The Int:Tir interaction induces the clustering of Tir leading to an intimate attachment of the bacteria. Tir is activated and recruits cellular proteins causing the polymerisation of filamentous actin at the site of bacterial attachment referred to as actin pedestals.

The comparison of the intimin proteins of EPEC and EHEC[214,399] reveals high sequence homology except for the C-terminal region, that shares only 49% identity. This region is the responsible to bind TirM with high

affinity, indicating that the different intimin sequences could confer different colonization patterns within the intestine. The interaction of intimin and Tir seems to induce the clustering of Tir[357], what is critical for downstream signalling towards efficient actin polymerization[171] (Figure 2). Activated Tir serves as a signalling effector that initiates the recruitment of host adaptors and actin nucleators from the host cytosol. Interestingly, A/E pathogens use divergent mechanisms of signalling. These paths converge on the activation of the actin nucleation promoting factor Neural Wiskott–Aldrich Syndrome Protein (N-WASP), which, in turn, recruits and activates the actin-related protein 2/3 (ARP2/3) complex (Figure 4). ARP2/3 is the responsible for the actin filament nucleation and pedestal formation[162] (Figure 3).

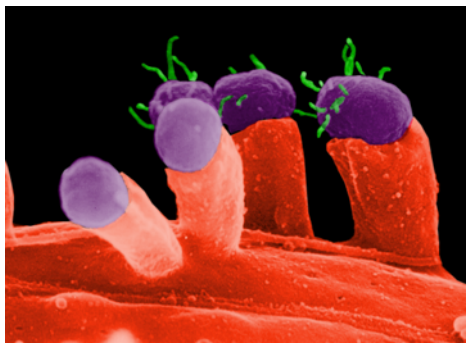


Figure 3. Artificially coloured image of scanning electron microscope showing EPEC on pedestals during the infection of an epithelial cell. The filaments of the T3SS (in green) surround EPEC (in purple) at the infection site. EPEC localises at the top of the formed actin pedestals (in red) in late infections. Adapted from Knutton *et al.*, 1998

iii) Alteration of cell homeostasis. Once the bacteria are intimately attached, they remain mostly extracellular in the lumen of the intestine and shorten the EspA filaments from their surface[251], but continue translocating effectors through the T3SS. As happens with Tir, the other effectors interact in the bacterial cytoplasm with LEE-encoded chaperones (CesT[213], CesF[90]), which prevent them from premature folding and facilitate their recognition and secretion by the T3SS[89,90,349]. Inside the host cell, the effectors subvert many host cell processes, leading to electrolyte loss and eventual cell death[73,290] and enabling the bacteria to multiply, better colonize and cause disease. A group of 21 core effectors is shared by all A/E pathogens, six of them inside the LEE (Tir, EspF, Map, EspG, EspH, EspZ)[78]. In addition, the pore-forming protein EspB - encoded in the LEE - is known to have an effector function over host myosin filaments[152]. However, the repertoire of the remaining non-LEE encoded (Nle) effectors[151] varies widely in different EPEC, EHEC and *C. rodentium* in a strain dependent manner, suggesting that different isolates employ specific infection strategies.

Many of the translocated effectors have multiple targets and function coordinately, indicating that the pathogens have evolved strategies to develop a succesful infection. Among the LEE-encoded effectors, Map controls actin dynamics to form transient filopodia[172], disrupts the structure of the mitochondria[269] and promotes intestinal hyperplasia[329]. EspF is targeted to the mitochondria where it triggers the apoptosis pathway[260,408] and directs its localization to the nucleolus[75]. In addition, EspF has been implicated in inhibiting phagocytosis[285] and hijacking the membrane trafficking[4]. It also disorganizes occludin leading to the disruption of apical tight junctions and the loss of transepithelial electrical resistance[129,236] in association with Map and EspG[121,230]. EspG on its own is capable of disrupting the secretion of the host cells by affecting the microtubule organization[121] and the Golgi[57], while EspH elongates actin pedestals, induces

cytotoxicity and cell detachment and inhibits phagocytosis[86,390,392] in association with EspF and the translocator EspB[152]. Interestingly, EspZ acts as regulator of the T3SS-dependent translocation by blocking the translocation derived from succesive infection of the pathogen to the same cell[24]. Nle effectors also play a role in the virulence of the pathogen, being implicated in reducing the protein trafficking[173,230] or disrupting the tight junctions[348]. However, they are mainly involved in the modulation of the immune response induced by the infection. They inhibit opsonophagocytosis[225] or block the host antimicrobial response either by interfering with specific nodes of the NF-kB immune response pathway[16,257], arresting the cell cicle[311] and/or antagonizing the apoptotic pathways derived from the infection[30,135,272,312].

EPEC O127:H6, strain E2348/69,

Enteropathogenic *E. coli* (EPEC)[92] is one of the leading causes of severe diarrheal illness in developing countries associated with high rates of morbidity and mortality in children[251]. EPEC was the first pathotype of *E. coli* to be described: a group of serologically distinct *E. coli* strains were isolated from children with diarrhoea but not from healthy children during large outbreaks of infant diarrhoea in the United Kingdom in 1945[37]. The reservoir of EPEC infection is thought to be asymptomatic adult carriers, including mothers and persons who take care of children, and symptomatic or asymptomatic children. In some studies, EPEC has been detected in up to a 20% of healthy infants younger than 2 years[202]. EPEC transmission is fecal-oral, through contaminated hands, weaning foods or fomites and the infection is produced in the small bowel. The main adherence factor for the infection is the bundle-forming pilus (BFP)[120], which is encoded on the plasmid pMAR2. BFP tend to aggregate and form bundles that mediate interbacterial adherence to form microcolonies. They also interact with *N*-acetyl-lactosamine-containing receptors on host cell surfaces[148]. The 385 kDa protein called lymphostatin (LifA) is thought to be another specific adhesion factor[11].

Using *in vitro* cell cultures, it has been shown that a 12-residue peptide of TirC is recognised by multiple redundant host tyrosine kinases[194], which phosphorilate Y474 activating the recruitment of the host adaptor protein Nck[45] to initiate pedestal formation *in vitro*[171]. Nck subsequently interacts with N-WASP through three SH3 domains[162] (Figure 4). Minor Nck-independent mechanisms reflect redundancy in the process of pedestal formation. Most of this activity requires Y474[46,87], reaffirming its role as a key residue for triggering actin assembly by EPEC Tir, but even in the absence of Y474, EPEC has been shown to generate actin pedestals mediated by an additional tyrosine residue at position 454 of TirC.

EHEC O157:H7, strain EDL933

Enterohemorrhagic *E. coli* (EHEC)[277] poses a serious public health concern and is associated with food poisoning infection outbreaks in developed countries[251] that imply characteristic abdominal cramps and

bloody diarrhea, as well as the life-threatening hemolytic uremic syndrome (HUS)[165] - a combination of anemia, thrombocipenia and renal failure. The recognition of EHEC as a distinct class of pathogenic *E. coli* resulted from two key epidemiologic observations in 1983[166,295] that associated hemorrhagic colitis and HUS with food contamination. When adults and children are infected, EHEC localises in the distal ileum and the large bowel[278]. The main differential virulence factors of EHEC are the phage-encoded Shiga toxins (Stx)[251], composed of two major subunits. Upon infection, the pentameric B subunit binds the glycolipid globotriaosylceramide (Gb3) on the surface of Paneth cells in the intestinal mucosa[321], inducing membrane invaginations to facilitate internalization. Stx is then trafficked to the Golgi and the endoplasmic reticulum, where the A subunit is activated. It interrupts protein synthesis and induces apoptosis[165], leading to necrosis of the epithelium. The release of Stx out of the cell occurs through lambdoid phage-mediated lysis in response to DNA damage and the SOS response[356] and disseminates to other organs[313] reaching the Gb3-positive epithelial cells of the kidney, where they cause inflammation[6,251] promoting HUS. Stx also mediates local damage to blood vessels in the colon, which results in bloody diarrhoea and haemorrhagic colitis, among other symptoms[131]. The treatment of EHEC with antibiotics is problematic, because they may enhance the replication rates of the pathogen and the expression of *stx* genes. The lysis of the bacteria sets Stx free, thus exacerbating the cytotoxicity and the risk of developing HUS[347]. Thus, an effective treatment against the infection of humans has not been yet developed.

The principal reservoir of EHEC is the bovine intestinal tract[258], but unlike in humans, EHEC colonization in adult ruminants is asymptomatic[395] (with rates of colonization of 10 to 25%), since EHEC colonizes the recto-anal junction of cattle, where cells lack Gb3[254,284]. The ruminants shed EHEC in their feces and bovine manure can harbor viable EHEC for more than seven weeks[258]. Humans mainly acquire the pathogen by consuming contaminated foods of bovine origin such as meat or milk, but also cross-contaminated vegetables or sprouts[344]. A very low infectious dose for disease - 100 to 200 bacteria - has been estimated from outbreak investigations[251].

EHEC possesses 16 potential fimbria-like operons[210] that contribute to adherence. They include two long polar fimbriae (LPF)[355] - also involved in biofilm formation -, the haemorrhagic coli pilus (HCP)[397], the metalloprotease StcE[128] or the IgrA homologue adhesin (Iha)[231]. A main factor for the adhesion is Efa1[259] (homologous to EPEC LifA), encoded in the 92-kb pO157 plasmid of EHEC[39]. This plasmid harbours up to 100 ORFs including other virulence factors, like RTX - a toxin similar to the UPEC alpha-hemolysin[58]. EHEC injects around twice more effectors into host cells than EPEC, most of them are redundant[354], which may provide EHEC with an evolutionary advantage to outcompete other bacteria. EHEC and EPEC have also evolved different mechanisms to activate the actin polymerization machinery. TirC of EHEC is not tyrosine phosphorylated by the host cell[82], lacks the equivalent of EPEC Tir Y474 and does not require Nck for actin pedestal formation. A NPY sequence at residues 456-458 of EHEC Tir is critical for the actin

rearrangement[36]. Y458 corresponds to EPEC Tir Y454, which leads minor Nck-independent actin assembly pathways in EPEC. The NPY458 sequence (and likely the equivalent sequence of EPEC Tir as well) binds to the insulin receptor tyrosine kinase substrate (IRTKS) and the homologue of insulin receptor substrate protein of 53 kDa (IRSp53)[378]. Importantly, EHEC requires an additional effector for pedestal formation[82] to link the C-terminal regions of IRTKS and IRSp53 to N-WASP[378] (Figure 4). This effector is an EspF homologue located outside the LEE called Tir cytoskeleton-coupling protein (TccP)[47,114]. TccP is composed of repeating elements[44,113], each with two structurally and functionally distinct domains: a C-terminal recognized by IRTKS and IRSp53[1] and an N-terminal that binds and activates N-WASP[310].

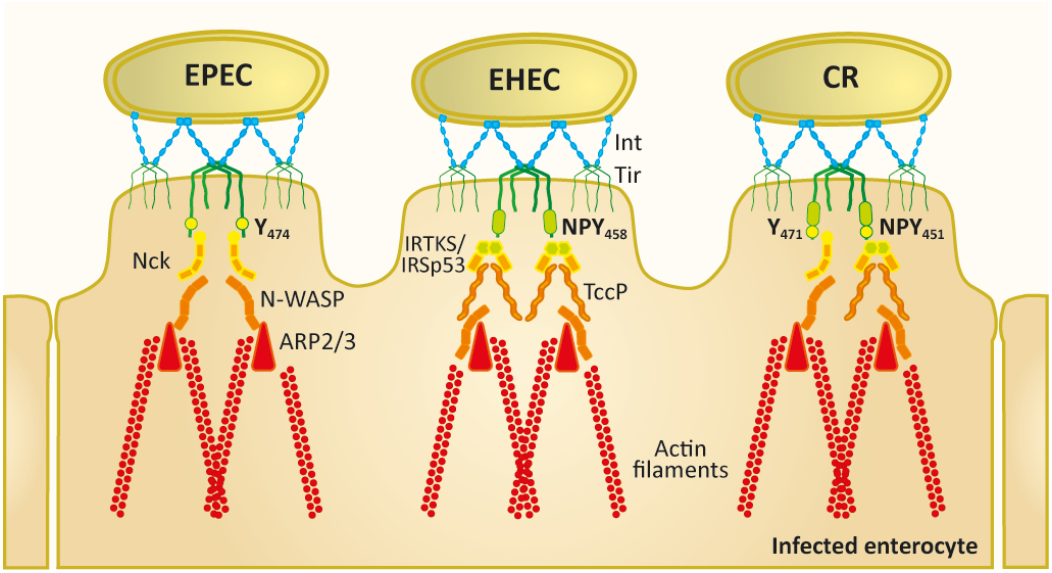


Figure 4. Main Tir signalling pathways in the formation of the A/E lesion. In the major pathway of EPEC the critical Tir residue Y474 is phosphorylated and recruits Nck. In contrast, the NPY458 motif of EHEC Tir recruits the host adaptors IRTKS and IRSp53, which in turn interact with the EHEC translocated effector TccP. In the case of CR, both pathways are activated by the homolog residues Y471 and NPY451. All the signalling cascades converge on common host actin nucleation factors, N-WASP and Arp2/3, to form actin pedestals.

Citrobacter rodentium (CR) O152, strain ICC169

CR is the agent of naturally occurring colitis in laboratory mice, also called transmissible murine colonic hyperplasia[247,316], what leads to a pronounced dysbiosis in the intestine. CR overgrows other intestinal bacteria[59,216] reaching 1-3% of the total intestinal microbiota and 10^9 colony forming units (CFU) per g in the colon[385]. Though, the infection is transient and few adult mice die from the symptoms. Most infection studies involve the inoculation of mice using an oral gavage with laboratory-cultured bacteria, which results in a highly reproducible infection cycle. Upon infection, adherent bacteria adapt to the gastrointestinal environment and undergo a virulence switch that facilitates colonization in the descending colon[386], provoking increased rigidity[247] and microscopic evidence of epithelial cell hyperplasia, including marked crypt elongation[59]. After a peak in the bacterial load 2 to 3 weeks postinfection, the infection begins to clear and bacteria are barely detected in the stool after 2 months[215].

Infections of mice with EPEC and EHEC isolates have been reported, but form no A/E lesions and do not colonise the intestinal mucosa, so the usefulness of these infection models is questionable. On the other hand, CR shares the mechanism of colonization with the human A/E pathogens and is thus arguably one of the best murine models of bacterial infection available, as it reflects a natural and physiological host-pathogen interaction in immunocompetent adult animals[247]. Both the Nck-dependent and independent pathways for the A/E lesion formation of EPEC and EHEC are present in CR (Figure 4) and there is also evidence of a third Tir-dependent pathway for actin polimerisation *in vivo*, what suggests that all these pathways are complementary in CR[64,79], possibly to reach a higher colonization advantage. Besides, virulence factors are generally interchangeable among A/E pathogens and CR can express them artificially without affecting to the infection rates. As an example, the EHEC effector TccP has been heterologously expressed in CR and it does not enhance its virulence[119]. A murine infection model CR that artificially produces Stx has also been generated to analyse the Stx-mediated disease in mice[222]. Hence, CR is generally used to assess the role of *E. coli* virulence factors in humans with predictive value.

Protein secretion systems of Gram-negative bacteria

The transport of proteins across the cell envelope is a basic function found in all groups of bacteria, which have evolved many sophisticated molecular machines to provide paths for the secretion of molecules and proteins needed for cellular functions (e.g. biogenesis of the cell envelope, adhesion or motility). However, pathogens also make use of them for the colonization of specific niches and the modulation of host responses (e.g. toxins and translocated effector proteins)[117,374].

The general secretory pathway (GSP) - also called Sec pathway - is the major mechanism for the secretion of proteins. It consists of a multi-subunit translocon (SecYEG) inserted into the bacterial inner membrane (IM) that forms a pore and recognises N-terminal signal peptides of unfolded proteins in the cytoplasm. An ATPase (SecA) energises the secretion along with the proton motive force[301]. Besides, certain proteins are transported by the Tat secretory pathway[62]. The GSP is sufficient for the secretion of proteins in Gram-positives, but Gram-negatives can only export proteins into the periplasm using this pathway, so are posed with the challenge to translocate molecules across the outer membrane (OM). The OM provides in turn a protective barrier against various antimicrobial host defenses as well as against antibiotics. To date Gram-negative bacteria are known to have evolved six main mechanisms to circumvent this problem[80] (Figure 5). The difference between these systems is in the components that form the transport machinery. Some of them secrete proteins from the periplasm, while the rest translocate proteins directly from the cytosol. In general, they recognise a conformational secretion signal rather than a specific primary sequence[102].

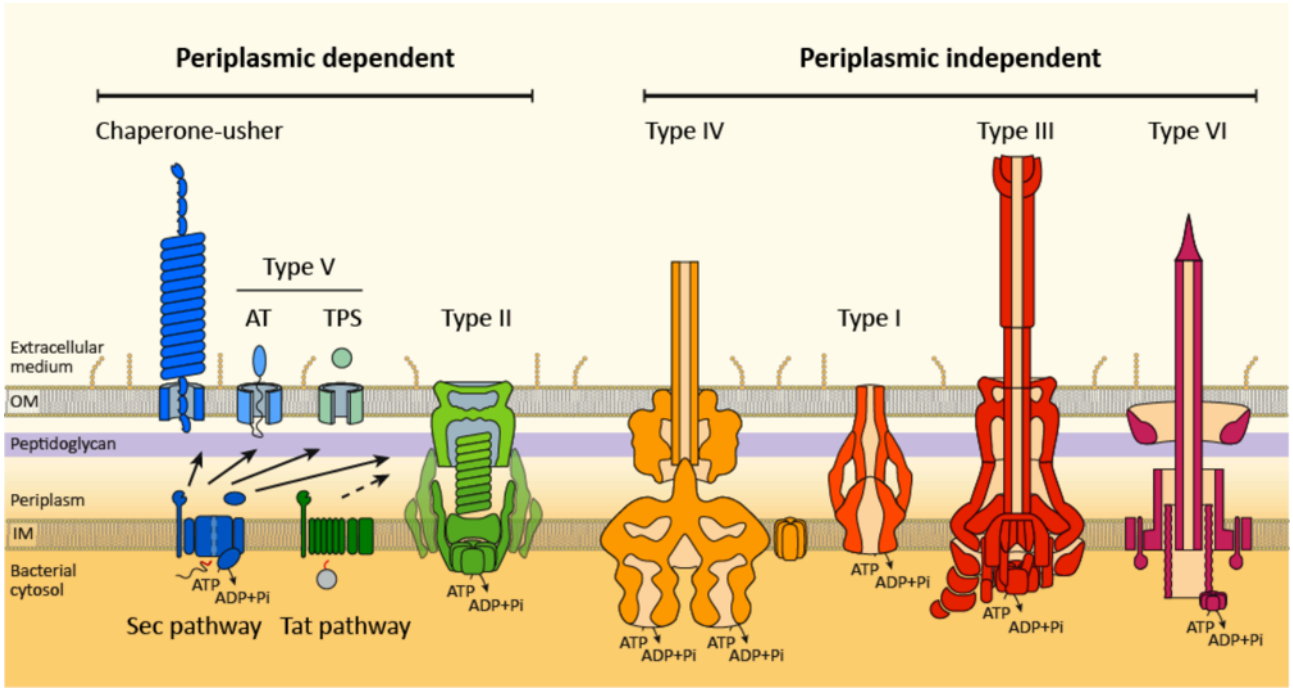


Figure 5. Secretion systems of Gram negative bacteria. The periplasmic dependent systems make use of the Sec (or Tat) pathways. The chaperone-usher pathway is responsible for the exposure of fimbrial adhesins, while the autotransporters (ATs) expose non-fimbrial adhesins. ATs and the two partner systems (TPS) belong to the T5SS, but the TPS secretes and not exposes proteins. The T2SS makes use of a pseudopilus to pump periplasmic proteins out. On the other hand the periplasmic independent secretion proteins recognise proteins of the cytoplasm. The Type IV and the Type III deliver proteins to eukayotic cells, while the Type VI targets other bacteria. On the other hand, the Type I translocates proteins to the medium.

Periplasmic dependent secretion systems

The analysis of a large number of bacterial genomes indicate that up to 17% of the genes in Proteobacteria genomes encode proteins initially exported to the periplasm by the GSP[20]. The secretion systems that require a periplasmic step make use of these exported proteins for subsequent transport across or insertion into the OM[268]. Among the periplasmic dependent systems, the Type II Secretion System (**T2SS**) is a dynamic structure that spans the IM and the OM. It is a ring-shaped complex that recognises the periplasmic proteins and forms a contractile pseudopilus that propells them through an OM secretin to the extracellular medium[184]. By contrast, the **chaperone/usher** pathway anchors the proteins to the OM forming pili or fimbriae and provides bacteria with the ability to adhere to host cells or to a wide variety of biotic and abiotic surfaces for bacterial survival[176]. The multiple subunits of fimbrial adhesins are translocated across the OM through a mechanism in which a periplasmic chaperone binds the pilin subunits and directs them to an assembly OM protein platform (the usher) that polymerises the nascent pilus in an ordered manner[314]. The type 1 fimbriae (fim) and *E. coli* common pili (Ecp or Mat) are the most conserved fimbrial structures assembled by the chaperone-usher pathway among both, commensal and pathogenic strains[291,396]. Genome analysis of *E. coli* K-12[31] and some pathogenic *E. coli* strains[277,379] have identified multiple operons encoding putative fimbriae, but also cryptic *E. coli* adhesins[180,300] that are only expressed under very specific conditions[181], what suggests the existence of adhesins with different roles and

specificities. In fact, *E. coli* codifies many non-fimbrial adhesins, such as the conserved gene *flu* (encoding for Antigen 43)[175], which are autotransporters (**ATs**) that belong to the so-called the Type V secretion systems (**T5SS**)[368]. ATs are composed of two functional units: a secreted "passenger" domain that harbors a specific activity (e.g. protease, adhesin), and a ß-barrel domain anchored in the OM that allows secretion of the passenger domain in concert with its insertion, assisted by the ß-barrel assembly machinery (BAM) complex[127]. The intimins and invasins family of adhesins also belong to the T5SS and, like the ATs, are composed of one single polypeptide with a ß-barrel domain and a "passenger" region that is displayed on the bacterial surface, albeit with an opposite topology to passengers and ß-barrel domains of ATs[127]. A third group of related non-fimbrial adhesins belong to the two partner systems (**TPS**), in which two distinct polypeptides form a tandem for secretion. The secreted polypeptide (TpsA) has structural similarity with the "passenger" domain of ATs, whereas the second partner (TpsB) is an OM ß-barrel protein having structural similarity to the OM protein subunit of the BAM-complex[155]. TpsB recognises TpsA in the periplasm and drives its secretion across the OM to the extracellular medium[154].

Periplasmic independent secretion systems

These secretion systems have in common that they secrete proteins directly from the bacterial cytoplasm (without a periplasmic intermediate) through a continuous protein conducting channel that spans the periplasmic space, connecting the inner and outer membranes[117]. For instance, the Type IV Secretion System (**T4SS**) is believed to have evolved from conjugation machineries and forms a large macromolecular complex[211] characterized for translocating virulence factors and single-stranded DNA[319]. It can even internalize DNA from the medium[332] allowing genome plasticity and adaptation to the changing environment[109]. The Type VI Secretion System (**T6SS**) is functionally analogous to a bacteriophage tail[273] and corresponds to a contractile sheath-like structure[199] located in the cytosol and attached to the cell envelope[18]. T6SS activity is dynamic (extension, contraction, and disassembly cycles)[101] and has been associated with either contact-dependent antagonistic[141] or outright bacteriocidal[17] behaviour towards heterologous bacterial species. In this work, we have employed two periplasmic-independent secretion systems: the Type I Secretion System (**T1SS**) of α-haemolysin (HlyA) from UPEC strains and the **T3SS** of EPEC, which are described more extensively here.

HlyA secretion system

The HlyA secretion system[140,350] was first described in UPEC strains[380] and serves as a paradigm of the T1SS[163]. Only three proteins are necessary to secrete their substrate to the extracellular medium (Figure 6). HlyA - a 110 kDa hemolytic toxin - is the natural substrate[138,338], while HlyB and HlyD are two dedicated IM proteins encoded in a single operon. The third protein, TolC, localizes in the OM and provides the exit to

the extracellular millieu. TolC is a multifunctional protein that also participates in the secretion of other toxins and factors associated to different IM proteins[182,183].

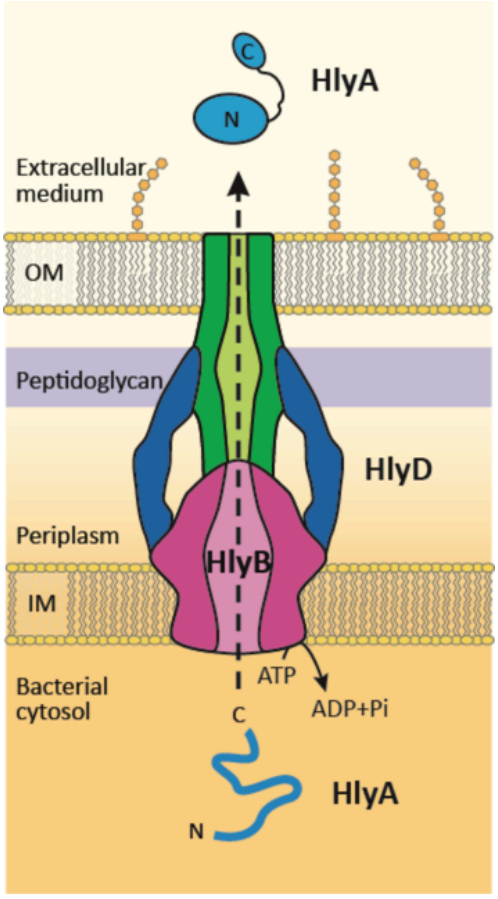


Figure 6. The HlyA secretion system. Three sole proteins transport hemolysin A (HlyA) from the cytoplasm to the extracellular medium. HlyB and HlyD recognise a C-terminal secretion signal and transport HlyA in an at least partially folded form across the cell envelope through the OM protein TolC.

The identification and characterization of the properties of the secretion signal of HlyA have been largely based on genetic analysis. It is composed of at least 46 residues of the C-terminal end of HlyA, is not removed during transport and contains a highly conserved, predicted amphiphathic helix[55], specifically required for secretion. The HlyA signal possesses no characteristic primary sequence motifs and there is no consensus sequence if compared with other T1SS[350]. The C-terminal location indicates that HlyA is translocated post-translationally and the size, charge and side chain residues at key positions, are sufficient for the specific recognition[140]. The signal interacts directly and specifically with HlyB, a dimeric ABC-transporter ATPase anchored to the IM with a cytosolic domain for the binding of ATP[318]. HlyA also interacts temporarily with a short 60 residue N-terminal cytoplasmic region of HlyD[12], a trimeric adaptor protein with a larger portion spanning much of the periplasm[320]. The binding of HlyA induces a conformational or chemical change in HlyD[12], triggering the recruitment of a TolC trimer, what creates a trans-envelope structure for the transport of HlyA. Trimeric TolC[182] forms a 12-stranded ß-barrel anchored in the OM by assembling four ß-strands of each monomer, and spans long α-helices towards the periplasm forming a hollow tube with a protein conducting channel. This channel remains closed until the interaction with HlyD promotes the hydrolysis of ATP in HlyB, generating the power stroke that moves HlyA across the bilayer. The α-helices of TolC widen the periplasmic entrance forming an open channel with a maximum diameter of 3 nm[5], what makes suitable the passage of only peptides with secondary structures[182], indicating that HlyA must be transported in an unfolded form. Interestingly, the large cavity at the external exit of TolC is thought to shelter the partial folding of the secreted substrates in a protected environment. HlyA is a 110 kDa protein, but substrates from other T1SSs vary from the relatively small (19kDa) HasA hemophore[200], to large surface bound proteins, such as adhesins or S-layer proteins, with molecular weights over 800 kDa in some cases[139]. The T1SS is also promiscuous, since a wide range of proteins can be secreted efficiently when engineered to carry the C-terminal secretion signal[106,323].

Type III secretion system

The T3SS[42] of EPEC is a very complex system built of 21 structural proteins and other accessory proteins, which assembles in a highly coordinately way spanning the cell envelope[32,191,227,262]. It is structurally and evolutionarily related to the flagellum[42,95], since its components share sequence and functional homology with the T3SS. Because of the shape and the ability to translocate proteins into the eukaryotic cytoplasm in a cell contact-dependent manner the T3SS is also called needle complex or injectisome. It is composed of a cylindrical basal body that anchors a needle-like structure to the bacterial envelope[38,61] (Figure 7). During infection, the bacteria receive external cues from the host environment to assemble the constituents of the T3SS, a process that must be coordinated in space and time[14]. The precise mechanism of T3SS assembly is still under investigation; however, it can be categorized into discrete stages: i) assembly of the basal body and the export apparatus, ii) assembly of the inner rod and the needle, iii) assembly of the filament and the translocon, and iv) secretion of effectors.

i) Assembly of the basal body and the export apparatus. The initial components are directed to the membrane by the GSP. The assembly begins as two separate building platforms. What it is called the export apparatus associates in the IM, where proteins EscR, EscS and EscT form a scaffold recognised by EscV[83]. EscV oligomerizes to form an export ring[83], but little is known about the structure or function of the other membrane components. On the OM, the secretin EscC also forms a ring complex[336] and leaves a periplasmic portion that is recognised by EscD[262], another ring forming protein. EscD subsequently recuits EscJ, a ring with a smaller diameter that is accommodated inside of EscD, forming the IM ring[401]. Despite the 3.5 MDa size of the needle complex, the rings formed only by EscC, EscD, and EscJ[336] draft its shape: a wide base and neck-like region with an internal channel spanning the cell envelope[167]. The autoprotease EscU interacts with EscV and couples the two assembly platforms into one structure. A cytosolic ring formed by EscQ binds to this structure and recruits the ATPase EscN[27], coupled to EscL in an inactive form[298]. The structure of EscN[405] depicts three domains: the N-terminal facilitates oligomerization into a homohexamer, a central ATPase domain binds ATP, and the C-terminal is the proposed recognition site of the substrates[334]. Binding of EscO to EscN promotes a change of conformation in EscL that activates EscN[298].

ii) Assembly of the inner rod and the needle. The conformational changes produced by the activation of EscN allow the assembly of the so-called inner rod[305], composed of EscI monomers. The inner rod is a periplasmic cylinder accommodated inside the basal body that forms a channel spanning the periplasmic space and serves as an anchor of the needle of the T3SS to the basal body. Based on predicted coiled-coil regions within the primary sequence, the EscI is proposed to be paralogous to the extracellular needle subunit (EscF)[265] and capable of self-polymerization[305]. A complex of EscF with two cochaperones (EscE and EscG)[306] is recognised by the ATPase and polimerises[387] extending the inner rod and forming a hollow

cylinder with an inner diameter of 2-3 nm, what indicates that the T3SS supports translocation of at least partially unfolded substrates[32]. EscP is anchored both to the needle tip and the basal body of the T3SS acting as a molecular ruler of the needle length[245] and signals a substrate specificity switch from needle components to translocator proteins to the basal body when the needle is fully elongated.

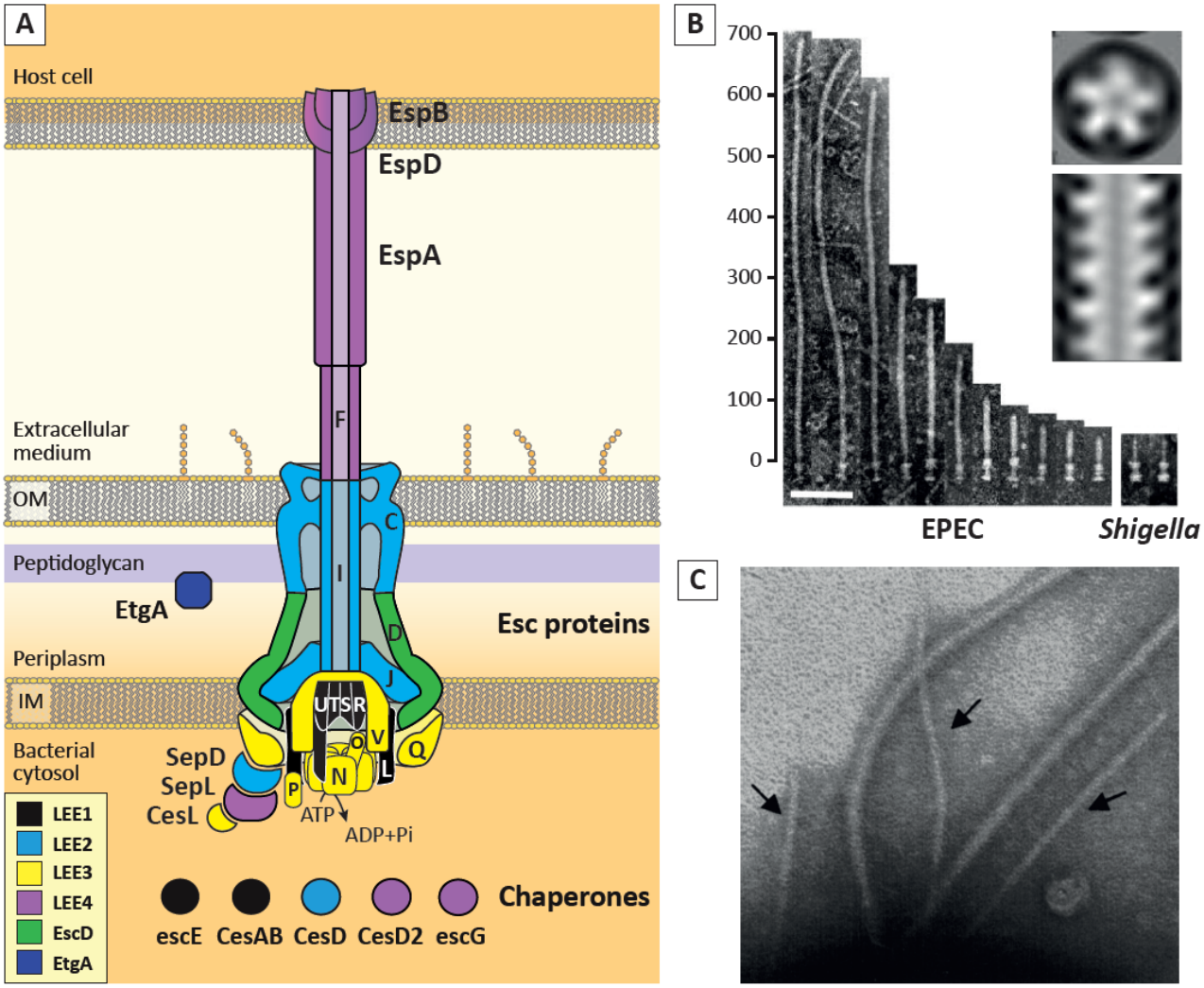


Figure 7. The structure of the T3SS of EPEC. A. The T3SS comprises a basal body anchored to the bacterial envelope and a filament attached to it. The many proteins that build it have specific structural functions or recognises proteins to help for their assembly on the structure or their secretion, as happens with the translocator proteins (EspA, EspB, EspD). B. The EspA filament is up to 700 nm long and has an internal channel through which the secreted proteins are thought to be transported. Electron micrographs are adapted from Sekiya *et al*., 1998 and Daniell *et al*., 2003. C. The T3SS can be observed on the cell membrane in electron micrographs (black arrows) and is similar to but thinner than the flagellum. Adapted from Daniell *et al., 2001*.

iii) Assembly of the filament and the translocon. EscU has a transmembrane domain connected to a C-terminal cytoplasmic autoprotease domain[351]. When the T3SS has assembled a fully formed needle, EscU suffers autoproteolysis[404] of its cytosolic domain, but it remains bound to the transmembrane domain. This rearrangement provokes a conformational change of EscU that leads to the switch of substrate specificity, thus controlling the timing of secretion. Then, participates in the recognition of the N-terminal secretion signals of the translocators EspA, EspB and EspD, coupled with specific chaperones (CesAB, CesD,

CesD2)[53,63,190,256]. These are the first translocated proteins by the T3SS, and can be detected in the supernatant of an EPEC culture. The distal end of the needle accomodates EspA, which multimerizes[400] into a filament that forms an extension of the needle[69,325] and can reach a length of more than 700 nm (Figure 7B). The EspA filament has a 2.5 nm diameter central channel[68] and likely plays a role in sensing contact with the host cell. On the other hand, EspB and EspD associate and form a heterooligomeric structure[212] that inserts preferentially inside lipid rafts[3] of the host cell membrane and forms a translocation pore[150] with an internal diameter of 1.2-3.5 nm.

iv) Secretion of effectors. It is thought that an uncharacterized signal senses contact with the host cell, altering the SepD, SepL and CesL[261,403] complex to promote a second switch of substrate specificity in the injectisome that stops the secretion of translocators. In their place, the effectors associated with chaperones CesT[286] or CesF start being recognised by their N-terminal (about 20 aminoacids) secretion signal[8] and injected into the host cell cytoplasm[241]. The hydrolysis of ATP by EscN energises the release of the chaperone from the unfolded effector and the subsequent loading of the unfolded substrate into the T3SS apparatus for translocation through the needle[289]. Another function thought for the effector-specific chaperones is to mask domains needed for membrane targeting within the host cell (e.g. Tir). Once translocated, the effectors seem to refold spontaneously in the cytoplasm of the host cell.

Single domain antibodies from camelids and intrabodies

Antibodies (Abs) are highly variable polypeptides produced by the B lymphocytes of the immune system and recognize virtually any molecular structure (i.e. antigens) identified as foreign with high specificity[96]. These characteristics make them valued molecules for medical and biotechnological purposes. Abs are large heterotetrameric proteins with Y shape composed of two bigger polypeptides termed heavy (H) chains and two smaller chains known as light (L) chains linked between them by disulphide bonds (Figure 8). Each chain is organized in immunoglobulin (Ig) domains and contains a variable domain (VH and VL respectively) in the N-terminal. All the variability of the Abs is located within the VH-VL pairs, and more specifically in a total of 6 hypervariable regions: the complementarity determining regions (CDRs), which together determine the specificity of the Ab for its cognate antigen.

Interestingly, members of the family *Camelidae* produce a special class of Abs devoid of light chains in addition to conventional Abs. In these heavy-chain-only Abs (HCAbs)[133] the antigen-binding site is formed by a single domain termed V_{HH} (VH of HCAbs) (Figure 8). The structure of the V_{HH} alone builds the antigen-binding surface of HCAbs, so *Camelidae* have adapted its amino acid sequence to gain variability, The V_{HH}s have extended CDRs able of adopting novel conformations[249] and recognize epitopes located in otherwise non-accesible clefts or protein cavities, such as active sites of enzymes[71] and inner regions of surface

proteins from pathogens[340]. To stabilize the hydrophobic core, they often have two additional cysteine residues (Cys) forming a disulphide bond[125]. These adaptations confer them strict monomeric behaviour, reversible folding properties, higher resistance to proteolysis and thermal degradation, when compared with the VH of conventional antibodies[366].

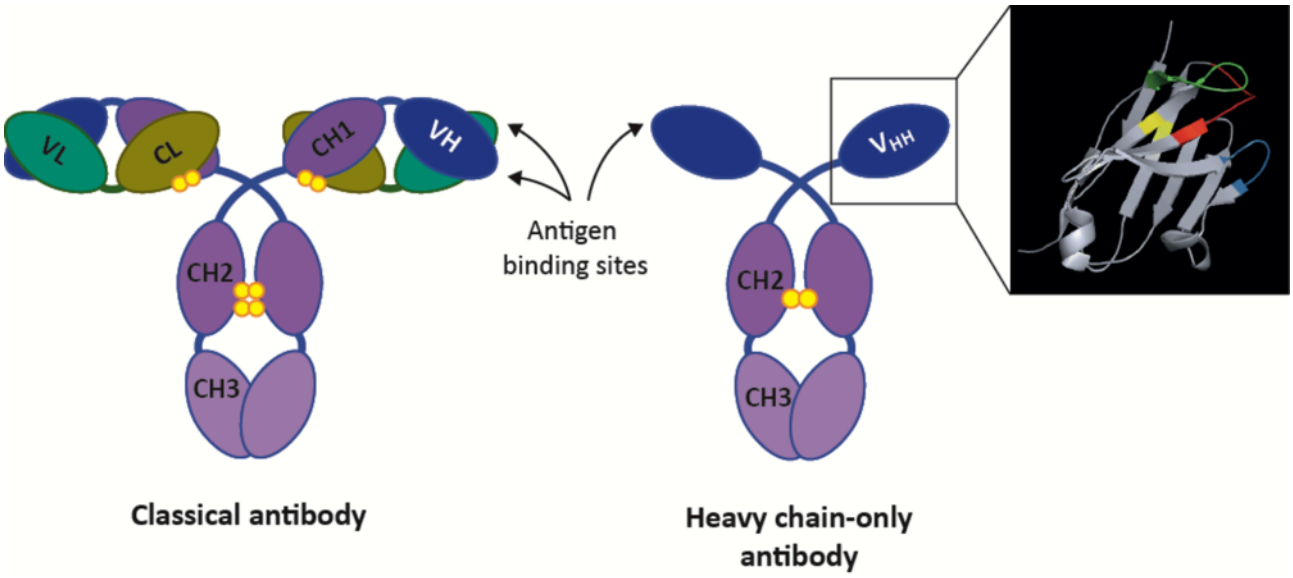


Figure 8. Camelid antibodies. Apart from the classical immunoglobulins, camelids codify Heavy chain-only antibodies (HCAbs) that lack the light chains. Instead of having two domains (VH-VL) responsible for the antigen recognisiton, one domain is enough for the HCAbs (V_{HH}). The three complementary determining regions of the V_{HH} domain (CDR1, in blue; CDR2, in red and CDR3, in green) form enlarged loops able to recognise otherwise hidden antigenic sites for the immune system. The disulphide bonds are marked in yellow.

These uncommon properties make the V_{HH}s very interesting in biotechnology. Their small size and the capacity to bind protein cavities leave open the possibility to use them as enzyme inhibitors[51] (or activators) or immunomodulators[292]. V_{HH} fragments, are the smallest recombinant Ab fragments generated to date (ca. 12-14 kDa). It has been demonstrated that, in addition to their small in size, their simplified structure allows the expression in microorganisms like bacteria [98] or yeast[97] and are highly stable soluble proteins[370] with a potent antigen-binding capacity. The recombinant expression of V_{HH}s yields a soluble single domain Ab (sdAb) fragment with dimensions of 2-4 nm, and has been also referred to as Nanobodies (Ablynx). In addition, the high similarity between V_{HH}s and human VH3 sequences open their potential use in therapeutic applications and *in vivo* imaging[134] derived from the short circulating half-lives in humans[255]. Selection of specific and high affinity V_{HH} binders against proteins of interest is an important step towards getting such applications. To do so, the standard methodology is Phage Display[98], in which a library of V_{HH} genes obtained from immunized animals is fused to the minor capsid pIII protein from filamentous bacteriophages (M13), allowing the display of the V_{HH}s on the surface of phage particles to generate the so-called Phage antibodies (Phabs). By means of a panning procedure in which the Phab library is incubated with an immobilized antigen on a solid support, Phabs carrying V_{HH}s against the specific antigen can be

selected and then amplified by infecting bacteria. After several panning rounds, individual Phab clones are isolated and the V_{HH}s on them identified by DNA sequencing.

At present, over 40 Abs have been approved for therapy by the Food and Drug Administration (FDA) of the US (www.antibodysociety.org/news/approved_mabs.php). Approved antibodies are designed for the treatment of several diseases such as cancer, autoimmune disorders or rare diseases[84]. Despite the general success of classical Abs in therapy, the large size of these molecules leads to practical drawbacks such as lower tumor or tissue penetration, hindering their use in applications like radioimmunotherapy and *in vivo* diagnostic imaging. Most recent Abs being approved and entering in clinical trials are humanised antibodies produced in transgenic mice[197,248]. Selected V-domains of both heavy and light chains are cloned in a genetic scaffold encoding constant domains of human antibodies to generate fully human antibody molecules[218]. In the case of V_{HH}s, the short serum-life due to renal clearance may limit their efficacy in therapeutic applications in the absence of further engineering. For instance, bispecific V_{HH}s recognizing long-lived serum proteins like albumin and the therapeutic target have been generated, thus resulting in increased half-lives[352,375]. In the last few years, the use of V_{HH}s for health applications is being rapidly developed (www.ablynx.com) and some clones[15] are already in advanced stages of clinical trials, but still none of them has been approved for therapy.

An emerging application of antibodies is their intracellular use (intrabodies) to block cellular signalling pathways or to modulate the activity of target intracellular proteins upon binding[205]. Intrabodies can block a certain domain of the target protein, while the rest of the protein retains its function. Besides, intrabodies can also act as activators[229] of a molecular process and possess a longer half-life than, for example, the interfering RNAs (iRNA)[205]. Genetic engineering makes possible the fusion of cellular localization signals to the intrabodies to direct them to specific organelles (such as the nucleus[402]) of the eukaryotic cell[34], what confers them many possible therapeutic applications. However, the reducing environment of the cytoplasm affects the formation of the disulphyde bonds and hinders the proper folding of the intrabodies, what makes them lose functionality and even present lower expression levels, lower solubility or a shorter half-life inside the cell. Recombinant Abs with the capacity to interfere with the function of diverse cellular processes have been generated[276], including cancer signalling pathways[149] and proteins related to neurodegeneration[217]. The high stability of the V_{HH}s allows them to better maintain the antigen binding capacities even in the reducing environment of the cytoplasm, what makes them more suitable for their use as intrabodies[366]. As examples, V_{HH}s capable of stimulating or inhibiting the activity of caspase-3 to modulate the cell apoptosis[235], V_{HH}s that block the Rev multimers of the HIV virus[274,371] or V_{HH}s that interfere with the gelsolin-actin interaction[365] have been selected. These examples show the potential of intracellular V_{HH} antibodies as therapeutic molecules.

The potential of the therapeutic use of bacteria

While commensal bacteria buffer possible infections in the human gut, the use of specific strains to actively combat pathogens has been widely studied. Pasteur's colleague Elie Metchnikoff first proposed the manipulation of the intestinal microbiota to improve human health. He suggested that *"the dependence of the intestinal microbes on the food makes it possible to adopt measures to modify the flora in our bodies and to replace the harmful microbes by useful microbes"*[239]. At that time Henry Tissier observed that children with diarrhoea presented low number of "bifid" bacteria in their stools that were, on the contrary, abundant in healthy children[353] and suggested that these bacteria could be administered to patients to restore a balanced microbiota. Natural probiotic strains are thought to enhance the immune response in the intestine and prevent the colonization of intestinal pathogens and are a suitable alternative for the treatment of gastrointestinal affections[49]. The best-known examples belong to the Gram-positive bacteria *Lactobacillus* and *Bifidobacterium*, which can be administered as supplements in yogurt and other dairy products[9,142]. Regarding Gram-negative bacteria, the most broadly used strain is *E. coli* Nissle 1917 (EcN)[19], isolated by the army surgeon Alfred Nissle from the feces of a soldier during the First World War who, unlike his comrades, did not develop infectious diarrhoea during an outbreak of *Shigella*. At present, EcN is contained in a drug called Mutaflor and is used for the treatment of infectious diarrheal diseases[223], inflammatory bowel disease[189,322] and ulcerative colitis[126,326]. Furthermore, EcN has been administered to neonates to colonize their digestive tract and protect against multidrug-resistant pathogens[206]. Another probiotic *E. coli* strain is ABU 83972, used in therapies against recurrent urinary tract infections[341]. It was isolated from a patient of asymptomatic bacteriuria[203] and protects the urinary tract from the infection of other pathogenic bacteria[341]. ABU 83972 is related to UPEC, but expresses less virulence factors[85], maybe owing its low pathogenicity to some deletions and point mutations in its genome[406].

Current molecular technologies allow us to actively engineer bacteria for the benefit of human health. Bacteria can be modified to produce proteins and other molecules of biomedical interest, such as human insulin[122], interleukins[346] or antibodies[43], but more interestingly, they have great potential for the delivery of desired therapeutic molecules *in vivo*, in specific organs or tissues of the animal, where they can produce a continuous supply. For instance, *Lactococcus lactis* was engineered to produce interleukin-10 (IL-10) in the intestine, which regulates the mucosal immune system by preventing excessive inflammatory responses towards normal intestinal bacteria, as happens in Crohn's disease[35,145]. Antibody fragments against tumor necrosis factor-α (TNF-α) have been used as therapeutic molecules delivered *in situ* by *L. lactis*, resulting in a reduction of the inflammation rate of mice suffering from induced chronic colitis[369]. *Lactobacillus jensenii,* a microorganism that is part of the normal flora in the human vagina, was engineered to stably express cyanovirin-N, a cyanobacterial protein with antiviral activity against HIV[193]. The observed property of anaerobic and facultative anaerobic bacteria (such as *Salmonella enterica* and *E. coli*) to

naturally target and proliferate in solid tumors[105] prospects possible treatments using these bacteria. Upon systemic administration, bacteria direct to the anoxic necrotic areas of the tumors, where they can also proliferate protected from the action of the immune system[382]. It is thought that the bacteria could be sensing tumoral metabolic by-products[168] and that the leaky vasculature of the tumor could facilitate their entry[220]. These bacteria have been equipped with genes encoding toxins, such as cytolysin A[158] and pro-drug converting enzymes like herpes simplex virus thymidine kinase (HSV-TK)[271] or *E. coli* cytosine deaminase (CD)[174], which are able to convert a non-toxic compound or prodrug (i.e. ganciclovir, 5-Fluorocytosine) into a toxic drug (i.e. ganciclovir monophosphate, 5-Fluorouracil). In other studies, invasive bacteria have also been engineered to produce apoptotic factors[65,111,208] or cytokines to promote the immune response and reduce the tumor size[65,207]. Upon cell invasion, bacteria are lysed in the phagosomes releasing their total cellular content.

Currently, there are efforts to optimize the production of the therapeutics in engineered microorganisms using expression cassettes that could be activated in response to certain environmental signals[21]. These modules should be stably inherited through cell generations for the stability of the expression[280] and could be combined to produce new complex bacterial assemblies, without perturbing or being perturbed by other cellular functions. These advances leave open the possibility to adapt cellular devices (e.g. secretion systems) and metabolic pathways to specific needs. For instance, *E. coli* has been engineered to detect and move towards the quorum sensing molecules that are naturally secreted by the pathogen *Pseudomonas aeruginosa* and induce the secretion of both DNAseI and microcin S, to degrade its biofilms and kill the bacteria[147].

Engineering protein secretion systems for the delivery of therapeutic proteins

The protein secretion systems of Gram-negative bacteria can be engineered for a more specific delivery of therapeutic proteins. The fusion of polipeptides to the signal of the HlyA secretion system can be applied for the secretion of proteins to the culture supernatants for purification. In a first assay, the B subunit of an Stx was fused to the HlyA signal to be secreted by an attenuated *Salmonella* strain harbouring a vector encoding the HlyB and HlyD transporters[362]. We have previously demonstrated that recombinant antibodies can be secreted by a commensal *E. coli* strain harbouring these vectors in a properly folded and functional conformation[99,100]. Moreover, a V_{HH} with dimerization capacities recognising α-amylase was also secreted through this system[106]. The HlyA system has also been used for the generation of live vaccines based on the secretion inmunogenic determinants of certain pathogens[116], generating humoral and/or cell-mediated immune responses against these molecules. Interestingly, EcN has been modified to secrete a peptide of the membrane protein gp41 of the HIV fused to the HlyA signal[287]. The approaches made by these studies encourage the use of modified bacteria with the ability to secrete a wider repetoire of V_{HH}

sequences through the HlyA secretion system for the selection of sdAbs that could be applied for the treatment of infectious diseases or even the development of live vaccines.

On the other hand, we have previously shown that the V_{HH}s can be anchored on the OM of *E. coli* by fusing them to the ß-barrel domains of ATs and Intimin[226,279]. The anchored V_{HH}s serve as synthetic adhesins for the binding of the bacteria to abiotic and biotic surfaces, while some natural *E. coli* adhesins have been deleted[279]. More interestingly, we have demonstrated that an engineered strain harbouring a synthetic adhesin recognising GFP can be specifically targeted to GFP-expressing HeLa tumors in mice using lower infection doses than a strain lacking the synthetic adhesin[279]. However, these strains should be modified to produce a therapeutic effect in the tumors. Following this approach, we have developed a strategy for the delivery of V_{HH}s to the cytoplasm of infected cells. It has been demonstrated that an efficient secretion of fusions between the N-terminal signals of EPEC/EHEC effectors (e.g. EspF, Tir, Map) and the enzyme ß-lactamase only require the first 20 residues of the effectors[52]. Once in the host cell cytoplasm, the enzyme is capable of catalizing the hydrolisis of the fluorescent compound CCF2/AM, indicating that the fusion protein has been translocated[52]. Thus, we fused V_{HH} sequences binding GFP or a-amylase to the N-terminal 20 amino acid T3 signal of EspF from EPEC, and showed that attenuated EPEC strains harbouring vectors with these constructs can secrete these model V_{HH}s to the culture supernatants in a functional form and inject them into the cytoplasm of HeLa cells through the T3SS[29]. In other studies, the T3SS of *Salmonella* has been used to secrete monomers of the spider silk protein[384] or to inject certain antigens to mammalian cells that stimulate the immune response in the host after injection[267,303,360,409], and the T3SS of *Pseudomonas aeruginosa* has been engineered to secrete antigens or nucleases using attenuated strains[94,157,196]. These results represent a proof-of-principle of the capacity of *E. coli* bacteria to directly deliver intrabodies into human cells. However, the use of pathogenic strains for the delivery of intrabodies is inconvenient for the development of feasible treatments, so the generation of non-pathogenic strains harbouring a functional T3SS capable of injecting intrabodies or even other therapeutic molecules is desirable to reach this objective.

OBJECTIVES

This work is based on previous results from our laboratory showing that V_{HH}s can be secreted by *E. coli* K-12 using the HlyA secretion system[106]. These sdAbs have high stability, extraordinary binding properties, and are suitable for human therapy. Thus, we wondered if we could engineer a non-pathogenic *E. coli* strain to secrete V_{HH}s in the gastrointestinal tract against a bacterial pathogen (e.g. EHEC O157:H7) with the aim to deliver these molecules *in situ* and passively protect susceptible hosts from the infection. In addition, previous studies in our laboratory had demonstrated that V_{HH}s could be injected to HeLa cells by EPEC strains carrying a functional T3SS[29]. However, the use of a pathogenic *E. coli* strain - such as EPEC - for the injection precludes the development of this technology for clinical applications. We hypothesize that functional T3SS from EPEC could be expressed in a controlled manner by a non-pathogenic *E. coli* strain such as K-12. Hence, the specific objectives of this thesis were:

1. To select and characterise V_{HH} antibodies of high affinity and specificity against essential proteins for the infection of EHEC O157:H7 (i.e., EspA, Int280, TirM) that could act as potential inhibitors of the intimate attachment of EHEC to intestinal epithelial cells during infection.

2. To engineer the secretion of selected V_{HH}s against EHEC O157:H7 in *E. coli* K-12 using the HlyA secretion system and to establish an *in vivo* mouse model that would allow us to test the activity of the selected V_{HH}s or the engineered *E. coli* K-12 strains during the infection of an A/E pathogen.

3. To integrate all genes required for the assembly of functional EPEC T3SS injectisomes in the chromosome of *E. coli* K-12 under the control of inducible promoters and to test their expression and the assembly of injectisomes in the *E. coli* K-12 cell envelope.

4. To evaluate the functionality of the injectisomes expressed by *E. coli* K-12 for the translocation of proteins into human cells.

Este trabajo se basa en resultados previos de nuestro laboratorio que muestran que los V_{HH}S se pueden secretar usando *E. coli* K-12 mediante el sistema de secreción de HlyA[106]. Estos sdAbs tiene alta estabilidad y propiedades de unión expeciales, lo que les hace atractivos para su secreción en el tracto gastrointestinal y así combatir patógenos bacterianos (como EHEC O157:H7). De esta forma, estas moléculas se podrían distribuir *in situ* para proteger a posibles hospedadores de la infección. Además, estudios previos del laboratorio habían demostrado que cepas de EPEC con un T3SS funcional pueden inyectar V_{HH}s a la células HeLa[29]. Sin embargo, el uso de una cepa patógena como EPEC para la inyección complica el desarrollo clínico de aplicaciones. Nosotros planteamos que el T3SS funcional de EPEC podría expresarse de forma controlada usando cepas K-12 no patógenas. Con todo, estos fueron los objetivos específicos:

1. Seleccionar y caracterizar anticuerpos V_{HH} de alta afinidad y especificidad contra proteínas esenciales para la infección de EHEC O157:H7 (EspA, Int280, TirM) capaces de actuar como posibles inhibidores de la unión íntima de EHEC a las células del epitelio intestinal durante la infección.

2. Diseñar una cepa de *E. coli* K-12 para que secrete mediante el sistema de secreción de HlyA anticuerpos V_{HH} contra EHEC O157:H7 y establecer un modelo en ratón para probar la actividad de los V_{HH}s seleccionados y de la cepa *E. coli* K-12 diseñada en infecciones de un patógeno A/E *in vivo*.

3. Integar todos los genes requeridos para ensamblar inyectisomas funcionales del T3SS de EPEC en el cromosoma de *E. coli* K-12 bajo el control de promotores inducibles y probar la expresión y el ensamblaje de inyectisomas en la membrana de *E. coli* K-12.

4. Evaluar la translocación de proteínas a células humanas mediante los inyectisomas expresados por la cepa comensal *E. coli* K-12.

MATERIALS AND METHODS

1. Conditions for bacterial growth

E. coli strains used in this work are listed in Table 1. Bacteria were grown at 37°C in Luria-Bertani (LB) agar plates (1.5% w/v)[240], in liquid LB medium or in DMEM, unless otherwhise indicated. When needed for plasmid or strain selection, antibiotics were added at the following concentrations: ampicillin (Ap) 150 µg/ml; chloramphenicol (Cm) 30 µg/ml; kanamycin (Km) 50 µg/ml; tetracycline (Tc) 10 µg/ml; spectinomycin (Sp) 50 µg/ml; apramycin (Apram) 50 µg/ml, except for strains expressing the Ap resistance gene from their chromosome, which were selected with Ap at 75 µg/ml.

1.1. Protein secretion with the Hly secretion system

Cultures of *E. coli* strain HB2151 carrying the indicated pEHlyA derivatives and pVDL9.3 (*hlyBhlyD*), or a pVDL9.3-derivative with the indicated V_{HH}-HlyA fusion were grown overnight (o/n) at 30°C in liquid LB containing 2% w/v glucose (to repress the *lac* promoter) and the appropriated antibiotics for plasmid selection (Ap and/or Cm). Next, bacteria were inoculated in fresh medium lacking glucose and grown at 30°C until OD_{600} reached 0.5. At this point, bacteria were induced with 1 mM isopropyl-1-thio-b-D-galactoside (IPTG) and further incubated for 6 h with gentle agitation (100 rpm). Cultures were centrifuged twice (10 min, 10000 g, 4°C) and the supernatants were equilibrated to PBS 1x (by adding 1/10th volume of PBS 10x) and used for ELISA, Western blot or purification of the His-tagged V_{HH}-HlyA fusions.

In the case of strain HS-TD4, o/n cultures grown at 30°C in liquid LB were inoculated in fresh LB and further grown until OD_{600} reached 0.5. At this point, bacteria were induced with 0.4% arabinose and incubated for another 4 h with gentle agitation (100 rpm). The culture supernatants were precessed and analyzed as above.

1.2. Induction of the T3SS in EPEC, EHEC, CR and SIEC

EPEC strains were grown o/n with agitation (200 rpm) at 37°C in a flask with 10 ml of liquid LB. Next day, cultures were inoculated in capped Falcon tubes (BD Biosciences) with 5 ml DMEM and incubated in static conditions for 2.5 h for the expression of the T3SS. These cultures were used at this point to infect cell cultures. Alternatively, for the analysis of the T3 secreted proteins, the cultures were grown for additional 3.5 h in DMEM. For the secretion of V_{HH}s with the T3SS, the DMEM cultures were induced with 0.1 mM IPTG after reaching OD_{600} 0.4 and further incubated 4 h.

EHEC and CR strains were grown 8 h with agitation (200 rpm) in a flask with 10 ml of LB-broth, inoculated in capped Falcon tubes with 10 ml DMEM and further incubated o/n in static conditions for the induction of the T3SS. These o/n cultures were directly used for the infection with the indicated dilution.

For the analysis of T3-secreted proteins by SIEC strains, bacteria were grown in capped Falcon tubes with 5 ml of liquid LB and 0.1 mM IPTG with agitation (160 rpm) for 6 h. For the infection of cell cultures with SIEC strains, bacteria were grown as above for 2.5 h and then added to mammalian cell cultures in DMEM with 0.1 mM IPTG for 3 h, unless otherwise indicated.

2. DNA constructs

E. coli DH10B-T1^{R} strain was used as host for the cloning and propagation of plasmids with the pBR, ColE1 or pSC101-ts origins of replication. In the case of suicide pGE-plasmid derivatives - harbouring the R6K origin of replication - the *E. coli* strains BW25141 or CC118-λpir were used, which express the Pi protein, needed for the replication of the plasmid[337].

Plasmids employed in this study are summarized in Table 2. We used standard methods of DNA manipulation[10] and all DNA constructs were verified by sequencing (Secugen, StabVida). Oligonucleotides (Table 3) were synthesized by Sigma. PCRs were performed with the Taq DNA polymerase (Roche, NZyTech) for standard amplifications in screenings and with the proofreading DNA polymerase Herculase II Fusion (Agilent Technologies) for cloning purposes. PCR products longer than 3 Kb were inserted in the pCR-BluntII-TOPO plasmid (Zero Blunt TOPO PCR Cloning Kit, Life Technologies) prior to the cloning in the final vector. Details of plasmid constructions are described below.

pEHlyA5 plasmids. To enhance secretion and purification of V_{HH}s, pEHlyA4SD was modified into pEHlyA5. A synthetic TD4-HlyA fusion harbouring a N-terminal 6xHis tag for purification and hemagglutinin (HA) and E-tags for detection was obtained using DNA synthesis (Geneart). This construct was cloned NcoI-BamHI into the pEHlyA4SD backbone. Clones IB10, EC7 and Vamy were then cloned SfiI-NotI into the pEHlyA5 backbone.

pVDL9.3 derivatives. Fusion proteins TD4-HlyA and Vamy-HlyA from pEhlyA5 plasmids were cloned BglII into the pVDL9.3 backbone. The correct direction of the insert was checked by PCR.

pT3sVgfp and pT3sVgfp-bla derivatives. VCapG2 and VCapG4 sequences were amplified by PCR and cloned SfiI-NotI substituting Vgfp. pT3sVgfp-bla derivatives harbour V_{HH} fusions with the TEM ß-lactamase lacking its Sec-dependent-signal peptide.

pET28a derivatives. Int280 (660-939 aas), EspA and TirM (252-360 aas) sequences of EHEC and TirM of EPEC (255-363 aas) and CR (253-360 aas) were amplified by PCR and cloned EcoRI-HindIII into the pET28a plasmid backbone under the T7 promoter and fused to a N-terminal His-tag for purification.

Integrative pGE and pGETS plasmids. Derivative plasmids of pGE and pGETS harbour integrative cassettes for the substitution or deletion of sequences in the bacterial chromosome. The homology regions (HRs) and the gene of interest (if present) of these plasmids were modified according with Figure 9 (unless otherwise indicated) resulting in different configurations depending on the purpose of the vector. Parting from plasmid pGEflu, a multicloning site (MCS) followed by the sequence of GFP^{TCD} was amplified by PCR and cloned SacI-SpeI. This plasmid was then used for the generation of the pGE plasmids harbouring other HRs and inserts, as described in Table 2. For the cloning of eLEEs, the respective integrative cassettes of the pGE plasmids were cloned into the pGETS backbone. In the case of eLEE1, its respective plasmid (harbouring *yfaL* HRs) was modified by hibridization with overlapping complementary oligos to add XmaI and NcoI restriction sites (given that eLEE1 harbours a SpeI restriction site in its sequence) and to remove its Ptac promoter. In the case of pGETSΔLEE5CR, the HRs were cloned XhoI-XbaI and HindIII-SphI, while the $LEE5_{EHEC}$ sequence was cloned XbaI-HindIII, due to the presence of a SpeI site inside the operon.

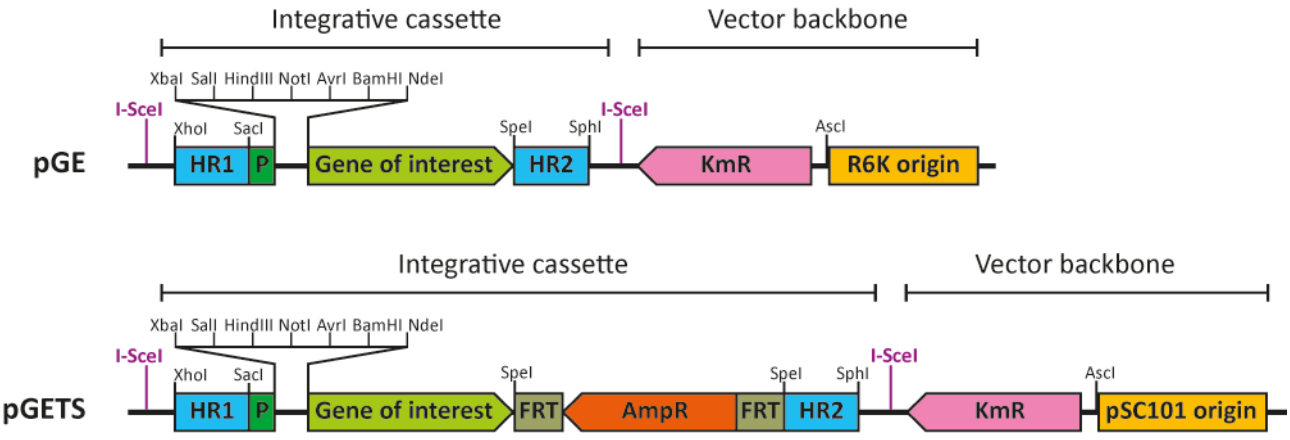


Figure 9. Plasmids used for the substitution and the deletion of genes from the genome. pGE and pGETS are suidice plasmids unless bacteria express the Pi protein or grow at 30°C, respectively. Two homology regions (HRs) recombine with the chromosome. In the case of substitution of genes, the genes of interest are cloned under the Ptac promoter (P) and between the HRs. For the positive selection of recombinants, the pGETS harbours an Amp resistance cassette flanked by FRT, which are prone to recombine in the presence of the flipase, thus eliminating the resistance cassette.

3. Edition of the bacterial genome (flow cytometry)

Some of the *E. coli* strains used in this work (Table 1) were generated by site-specific deletions and insertions in the chromosome of *E. coli*. They were originated with a strategy of genome edition based on expression of the λ Red genes[70] and the I-SceI endonuclease[281,282].

3.1. Use of suicide pGE plasmids with R6K origin of replication

The *E. coli* strain to be modified was initially transformed with plasmid pACBSR (Sp^R/Cm^R)[137], expressing the I-SceI and λ Red proteins under the control of the PBAD promoter (inducible with L-arabinose), and subsequently electroporated with the corresponding pGE-based suicide vector (Km^R). Colonies that grew in LB-Km-Cm/Sp plates had integrated the suicide plasmid in their genome (cointegrants) by recombination. Correct integration was verified by PCR. The colonies were grown for 6 h in LB-Cm/Sp liquid medium containing L-arabinose 0.4% (w/v) with agitation (200 rpm) to induce the expression of I-SceI and to cause a double-strand break in the genome that promotes a second step of recombination. A sample of these cultures was streaked on LB agar plates and incubated o/n to isolate individual colonies, which were replicated in LB and LB-Km agar plates to identify Km-sensitive colonies. The resolution of the cointegrates can either revert the sequence to the wt or maintain the mutant sequence, depending on the site of the crossover. Using specific primers (Table 3), the mutants - harbouring deletion, insertion or substitution of genes - were identified by PCR screening. Plasmid pACBSR was cured from the final strains by growth in liquid LB and streaking on LB agar plates. Individual colonies were replicated in LB and LB-Cm/Sp plates to screen for Cm/Sp-sensitive colonies. Construction details of individual *E. coli* strains are described in Table 4.

Those strains modified for the expression of GFP^{TCD} were subjected to cytometry analysis. To this end, grown liquid LB cultures were centrifuged. The pellets were washed with 500 μl of PBS and resuspended in a final volume of 1 ml in PBS. For each experiment at least 40000 cells were analyzed in the cytometer (Gallios, Beckman Coulter).

3.2. Use of suicide pGETS plasmids with thermosensitive pSC101 origin of replication

Those *E. coli* strains to be modified were transformed with plasmid pACBSR (Sp^R/Cm^R), as above, and the corresponding pGETS plasmid (Km^R) - which contains the thermosensitive origin of replication pSC101 - harbouring the Ap/Apram/Sp resistance marker flanked by Flippase Recognition Target (FRT) sites (Figure 9). The plates were incubated o/n at 30°C. Individual colonies were grown at 30°C in liquid LB medium with agitation (200 rpm) until reaching OD_{600} 0.5, we added L-arabinose 0.4% (w/v) to induce the expression of I-SceI and the λ Red proteins and shifted the temperature to 37°C to avoid the replication of the plasmid. The cultures were further grown during 4 h and streaked in LB-Ap/Apram/Sp plates - or LB-Ap/Apram/Sp-Cm/Sp to maintain the selection of the pACBSR plasmid - to select the mutant strains that had deleted, inserted or substituted the targeted genes. The grown colonies were analyzed by PCR with specific

oligonucleotides to check the correct insertions. Construction details of individual *E. coli* strains are described in Table 5.

To excise the Ap/Apram/Sp gene by te recombination of the FRT sites, the strains were transformed with plasmid pCP20 (Cm^R)[54], a thermosensitive plasmid that expresses flippase recombinase under thermal induction. Individual colonies carrying pCP20 were grown o/n at 30°C and inoculated in LB liquid medium at 37°C with agitation (160 rpm) to induce during 6 h. Thermal induction at 37°C of the flippase recombinase also inhibits the replication of pCP20, so individual Ap-sensitive colonies were screened by PCR to further confirm the modification of the genome.

4. Protein purification

Cultures of *E. coli* BL21(DE3) carrying the corresponding pET28a-derivative plasmid were grown at 30°C in 500 ml of LB and induced at OD_{600} 0.5 with 1 mM IPTG during 2 h. Cells were harvested by centrifugation (10000 g, 10 min, 4°C), resupended in 20 ml of Solution A - $NaPO_4$ pH 7, 300 mM NaCl, DNase (0.1 mg/ml; Roche) and protease inhibitor cocktail (Roche) – and lysed by passing three times through a French-Press at 1200 psi. The resultant lysate was ultracentrifuged 60 min at 4°C (40000 g) to obtain a cleared lysate supernatant. For purification of the His-tagged $Int280_{EHEC}$, $EspA_{EHEC}$, $TirM_{EHEC}$, $TirM_{EPEC}$ or $TirM_{CR}$, the lysates were passed through 2 ml of pre-equilibrated Cobalt-containing resin (Clontech) in a chromatography column and were washed with HEPES buffer (20 mM HEPES pH 7.4, 200 mM NaCl). The bound His-tagged proteins were eluted adding the same buffer complemented with 100 mM imidazole and were collected in 0.5 ml aliquots.

For the purification of the secreted His-tagged V_{HH}-HlyA fusions, supernatants from induced cultures were equilibrated with PBS and loaded at ca. 1 ml/min onto chromatography colums with pre-equilibrated Cobalt-containing resin (Clontech). Colums were washed with PBS or HEPES buffer and eluted with the same buffer containing 100 mM imidazole, as above.

A second purification step by gel filtration was performed for His-tagged antigens and V_{HH}-HlyA fusions used in Surface Plasmon Resonance (SPR) and/or immunization. Fractions eluted from metal-affinity chromatography were dialysed against HEPES-buffer (sterile filtered and degassed) and concentrated to 2 ml in a 3 kDa centrifugal filter unit (Amicon Ultra-15, Millipore). Next, protein samples were loaded onto a calibrated gel filtration column (HiLoad 16/600 Superdex 75, GE Healthcare), pre-equilibrated with HEPES-buffer. The elution was done using HEPES buffer and collecting 1 ml fractions. Protein concentration was estimated using the Bicinchoninic acid protein assay kit (Thermo Scientific).

5. Camel immunization and Phage display

5.1. Immunization and generation of the V_{HH} library

Purified His-tagged Int280, TirM and EspA from EHEC were diluted in sterile water and mixed at 2 mg/ml to the same volume of adjuvant (Veterinary Vaccine Adjuvant, GERBU) reaching a total volume of 5 ml. This solution was injected subcutaneously to one 14-years-old male dromedary camel (*Camelus dromedarius*). After the initial immunization, four boosting immunizations were performed in the same manner once per week (Figure 10). Pre-immune serum was prepared from a small blood sample (5 ml) before the first injection. The immune serum was collected seven days after the last immunization.. Serial dilutions of pre-immune and immune sera were used in ELISA to confirm the immune response against the respective antigens with protein A-POD as secondary. Additional 50 ml of blood of the immunized animal were collected from the jugular vein in tubes containing EDTA as anticoagulant, using Venoject system. For lymphocyte isolation, 50 ml of RPMI-1640 medium (Sigma) were added to the 50 ml blood sample and the final mixture was divided in 4 aliquots of 25 ml. Each aliquot was added on top of a 25 ml of Ficoll-Paque Plus (StemCell Technologies) in sterile capped 50 ml Falcon tubes. After centrifugation (800 g, 30 min, RT), lymphocytes were taken from the interphase, washed twice in RPMI-1640 by centrifugation (800 g, 10 min), resuspended in 5 ml of RPMI-1640, and the number of cells determined in a Neubauer hematocytometer (Hausser Scientific).

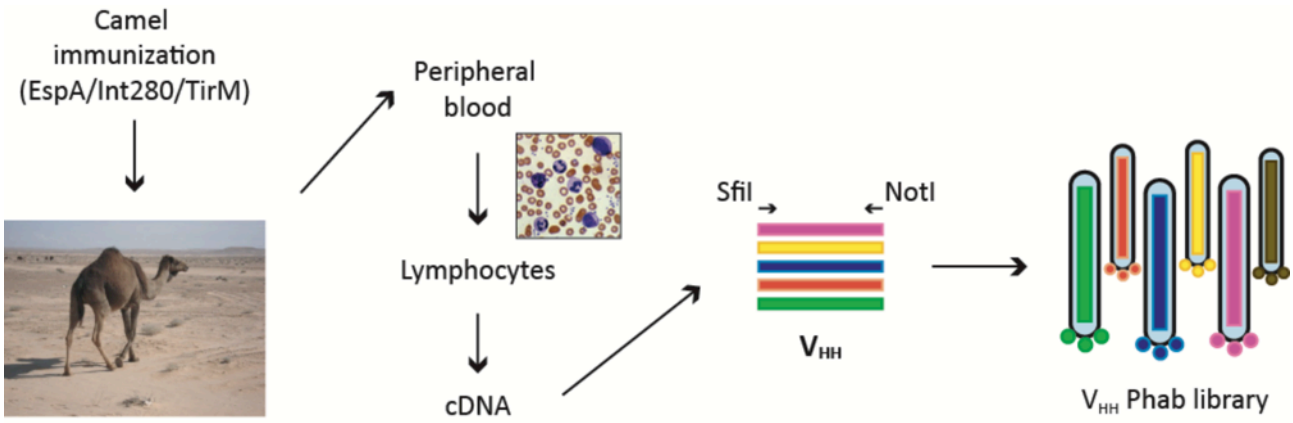


Figure 10. Generation of a V_{HH} Phab library. The purified antigens EspA, Int280 and TirM were inoculated in five injections to a camel. The lymphocytes were then isolated from its blood to extract the RNA. In a RT-PCR we synthesized the cDNA and in two subsequent PCR we amplified the V_{HH} domains that constituted the library. Those sequences were cloned in a pCANTAB5E plasmid to produced the Phabs that expose the V_{HH} domains on their surface.

About $2x10^7$ cells were lysed with 2 ml of Trizol (Invitrogen) for RNA extraction following manufacturer instructions. The poly-A^+ mRNA was purified from total RNA using an oligo-dT resin (Oligotex mRNA Minikit, Qiagen) and directly employed as template for first-strand cDNA synthesis reactions with random hexamers and oligo-dT primers (iScript cDNA Synthesis, Bio-Rad). About 1 µl of each cDNA synthesis was used as

template in 50 ml PCR reactions with oligonucleotides CALL001 and CALL002. The amplified fragments of ≈0.6 kb, corresponding to V_{HH}-C_H2 domains, and ≈0.9 kb, corresponding to conventional V_H-C_H1-C_H2 domains, were separated in 1.2% (w/v) low melting agarose gel and the ~0.6 kb band was purified (Qiaex II Gel Extraction kit, Qiagen). This fragment was used as template in a second PCR reaction with oligonucleotides V_{HH}-Sfi2 and V_{HH}-Not2 to finally obtain the amplified fragments of ~0.4 kb, corresponding to the V_{HH} domains. The amplified V_{HH} fragments were cloned SfiI-NotI into the phagemid pCANTAB5E backbone[232]. Ligations were electroporated in *E. coli* TG1 cells (Stratagene) and a library size of aprox. $2x10^6$ clones were determined by plating dilutions on LB-Ap agar plates with 2% w/v glucose at 30°C.

5.2. Packaging of Phabs into M13 particles

For preparation of phage antibodies (Phabs) a mixture of *E. coli* TG1 cells representing the library or a subpopulation after panning, was incubated in 25 ml of LB-Ap supplemented with 2% w/v glucose at 30°C until OD_{600} 0.2. At this point, 10^{10} plaque forming units (PFUs) of VCS-M13 helper phage (Km^R; Stratagene) were added for 1 h incubation at 37°C with gentle agitation. Then, *E. coli* cells were harvested by centrifugation (4000 g, 5 min) and resuspended in 250 ml of fresh LB-Ap-Km. After 16 h of incubation at 30°C, the cultures were chilled on ice and centrifuged (8000 g, 10 min at 4°C). To recover the M13-particles from the supernatant, 50 ml of PEG-NaCl solution (20% w/v polyethylen glycol 8000; 2.5 M NaCl) were added and the resulting mixture was kept on ice for additional 45 min. Phage pellets obtained after centrifugation (10000 g, 20 min at 4°C) were resuspended in 2 ml of TE (10 mM Tris-HCl, 1.0 mM EDTA, pH 8.0) and stored at -80°C.

5.3. Library enrichment by Phab panning

All steps were performed at room temperature (RT). Each antigen (10 µg/ml, in PBS) was adsorbed for 2 h to 4 wells (50 µl/well) of a microtiter immunoplate (Maxisorb, Nunc). These solutions were discarded and the wells were blocked by adding 200 µl/well of PBS with 3% w/v skimmed milk and 1% w/v BSA (Sigma). After 2 h, the blocking solution was replaced by a total of $2x10^{11}$ PFU of Phabs in 50 µl of PBS with 3% w/v skimmed milk. Phabs were allowed to bind for 1 h, and the unbound Phabs were removed from the plates by 20 washes of 1 min employing 200 µl/well of PBS with Tween20 0.05% and another 20 washes with PBS. To collect the bound Phabs, wells were incubated during 5 min with 0.1 M glycine pH 2.5 (50 µl/well). The solution from the wells was pooled together and immediately equilibrated by addition of one volume (400 µl) of 1 M Tris-HCl pH 7.5. Phabs in this solution were later used to infect TG1, which were plated on LB-Ap agar plates. After 24 h incubation at 30°C, colonies grown on these plates were harvested as a pool and used for phagemid packaging.

5.4. Selection of individual specific binders

For rapid screening of individual Phab binders, a small scale rescue of phagemids was performed in 150 µl cultures of TG1 grown in 96-well microtitre plates using 10^9 PFUs of helper-M13 phages. After production of the phagemids the plate was centrifuged (600 g, 10 min, RT) and the supernatants (containing the Phabs) were used in ELISA to determine their specific binding to the antigen.

At least $2x10^6$ independent colonies from each enriched Phab library were harvested from agar plates, and 50 units of OD_{600} were used for plasmid isolation (NucleoBond Xtra Midi, Macherey-Nagel). The V_{HH} fragments were cloned SfiI-NotI into the pEHlyA4SD backbone vector under the Ptac promoter and fused to the 200 aminoacids secretion signal of HlyA. The size of each library was $2\text{-}3\cdot10^6$ clones, as determined by plating dilutions on LB-Ap agar plates with 2% w/v glucose incubated at 30°C. Isolated plasmids from the pools were transformed in strain HB2151 harbouring plasmid pVDL9.3 and individual colonies were isolated in 96-well microtitre plates with 200 µl of liquid LB for the secretion of the V_{HH}-HlyA fusions.

6. SDS-PAGE and Western blot

Sodium Dodecyl Sulfate–Polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot were performed following standard methods[10] using the Miniprotean III system (Bio-Rad). Proteins separated by SDS-PAGE were either subjected to Coomassie Blue R-250 (Bio-Rad) staining, silver staining[7] or Western blot. For the latter, the proteins were transferred to a polyvinylidene difluoride membrane (PVDF, Immobilon-P, Millipore) using semi-dry electrophoresis (Bio-Rad), as previously described[10]. Antibodies employed for Western blot are described in Table 6. Membranes were developed by chemiluminiscence using the Clarity Western ECL Substrate kit (Bio-Rad) or a mixture of 100 mM Tris-HCl (pH 8.0) containing 1.25 mM luminol (Sigma), 0.22 mM cumaric acid (Sigma), and 0.0075% (v/v) H_2O_2 (Sigma). The membranes were then developed by exposure to X-ray films (Agfa).

6.1. T3SS proteins analysis

To analyze the secretion of T3SS components and the V_{HH}s in SIEC, EPEC and their derivatives, cells were harvested from 1 ml aliquots of the induced cultures by centrifugation (20000 g, 5 min). To obtain whole-cell protein extracts, the cells were resuspended in 400 µl of phosphate-buffered saline (PBS), mixed with 100 µl of 5X SDS-PAGE sample buffer and boiled for 10 min. For the analysis of the T3 secreted proteins, culture supernatants obtained after centrifugation were chilled on ice and incubated 60 min with trichloroacetic acid (TCA 20% w/v; Merck) for precipitation. After cold centrifugation (20000g, 15 min),

TCA-precipitated protein pellets were rinsed with cold acetone (-20°C) and resuspended in 30 µl of SDS-PAGE sample buffer for Coomassie staining or Western blot.

6.2. Synthesis and recognition of $TirM_{EHEC}$ peptides by TD4-HlyA

For the generation of 12-mer $TirM_{EHEC}$ peptides on a PVDF membrane it was used a MultiPep RSi synthesizer (Intavis) using the SPOT module (Proteomics Service, CNB-CSIC). The generated membrane was blocked in PBS containing 0.1% Tween 20 (PBST) and 3% (w/v) skimmed milk for 1 h at RT. The membrane was incubated with a solution of PBST with skimmed milk and the purified TD4-HlyA during 120 min and then washed with PBST. The procedure continued as a standard Western blot, with anti-E tag used as primary antibody and anti-mouse IgG conjugated with peroxidase (POD) (Sigma) as the secondary antibody.

7. Enzyme-linked immunosorbent assays (ELISAs)

ELISA conditions were based on those described previously[10,160]. Briefly, 96-well immunoplates (Maxisorp, Nunc) were coated for 120 min at RT with purified antigens (as indicated) diluted in PBS at a concentration of 5 µg/ml. Bovine serum albumin (BSA, Roche) was used as a negative control for detection. Phabs, culture supernatants or purified V_{HH}s were added to the wells at the indicated dilutions. For detection of bound Phabs, an anti-M13-HRP mAb (GE Healthcare) was added (1:5000). For detection of V_{HH}s, anti-E-tag mAb (1:2000; Phadia) and anti-mouse IgG-POD (1:2000; Sigma), as secondary antibody, were added. The reaction was developed with o-phenylenediamine (OPD, Sigma) and H_2O_2 (Sigma) and the OD_{490} was determined using a microplate reader (iMark ELISA plate reader, Bio-Rad).

For neutralisation assays, Int280 was previously biotynilated. N-hydroxide-succinamide biotin (NHS-Biotin, Sigma) was dissolved in DMSO. Proteins at 1 mg/ml were mixed with a ratio biotin:protein of 20:1. The solution was incubated 30 min at RT and then Tris-HCl 1 M pH 7.5 50 mM was added to incubate 1 h at 4°C. It was then centrifuged at 500 g in a 3 kDa centrifugal unit to concentrate the solution. Protein concentration was estimated using the Bicinchoninic acid protein assay kit (Thermo Scientific). For the assay, TirM was bound at 5 µg/ml to plastic 96 wells plates for 2 h. The wells were blocked with 3% (w/v) skimmed milk in PBS for 60 min. At the same time, biotynilated Int280 (50 µg/ml) was incubated with a 50 fold dilution of the camel immune or preimmune serums or 1 µM (50 µg/ml) of the corresponding purified V_{HH}s. These solutions were added to the wells for 1 h incubation after removing the blocking solution. Then, the wells were developed as a standard ELISA using streptavidin-POD.

8. Generation of rabbit antibodies

For the generation of antibodies against EscN, EscD and EscC, selected peptides of these proteins (Table 7) were synthesized using the FMOC strategy in a Multipep-Intavis AG (Proteomics Service, CNB-CSIC). All peptides were characterized by HPLC and Maldi-Tof mass spectrometry analysis. 2 mg of the peptides from EscD and EscC were conjugated to Keyhole Limpet Hemocyanin (KLH). The peptide from EscN was conjugated to the carrier protein albumin through glutaraldehyde (GA).

New Zealand White rabbits were kept in the animal house facility of the Medicine School of the Universidad Autónoma de Madrid. Prior to the injections, 300 μg of each conjugated peptide were diluted in 400 μl of 1 g/l NaCl solution and emulsified with 700 μl of Freund's complete adyuvant (Becton Dickinson) - for the first injection - or Freund's incomplete adjuvant - for the following ones. The injections were done subcutaneously each 3 weeks until the third injection. When needed, following injections were performed intradermically. Then, the blood of the rabbits was extracted 10 days after the last injection and left 5 h at RT to coagulate. Then, the blood was centrifuged (800 g, 10 min, RT) and the blood serum of the supernatant was stored at -80^{o}C. The immunoreactivity was determined by Western blot against whole cell extracts.

The serums were affinity purified against the corresponding peptides using 1 g of Tiopropil-Sepharose 6B resin (GE Healthcare) resuspended during 60 min in 3 ml of MilliQ water. The resin was washed with 200 ml of MilliQ water and 15 ml of Binding buffer (Tris 0,1 M pH 7.5, NaCl 0,5 M, EDTA 1 mM). 2 mg of the peptide were dissolved in 3 ml of Binding buffer and further incubated with the resin during 120 min with orbital agitation. The resin was introduced in the purification column, washed with binding buffer and with 3 volumes of AcH/AcNa 0,1 M pH 4 to block free radicals. The resin was then incubated 45 min with DTT 5 mM in that buffer and washed with 5 volumes of PBS. 1 ml of preimmune serum diluted 1/3 in PBS was passed through the column and the resin was washed again with PBS. It was then eluted with glycin-HCl 0,2 M pH 2,5 and washed again with PBS abundantly for equilibration. Then, the serum passed through the column followed by another wash with PBS. Next, the bound IgGs were eluted with glycun-Hcl 0,2 M pH 2,5 collecting fractions of 1,5 ml. These fractions were neutralized with Tris 1 M pH 8,8 and tested in a Western blot against whole cell protein extracts.

9. Surface Plasmon Resonance (SPR)

SPR experiments were performed using BiaCore3000 (GE Healthcare). All proteins solutions were dialyzed, sterile filtered and degassed at 4^{o}C during 2 h against HEPES-buffer (20 mM HEPES pH 7.4, 200 mM NaCl). Biotinylated $TirM_{EHEC}$ (as described above) at 0.1 μg/ml was immobilised on a Streptavidin SA chip (GE

Healthcare) at 150 response units (RU) at a flow rate of 10 μl/min in HEPES-buffer containing 0.005% (v/v) of the surfactant Polysorbate 20 (P20, GE Healthcare). For determination of binding kinetics, dilutions of purified TD4-HlyA or Int280 (as indicated) were flown at 30 μl/min in HEPES-buffer and sensograms were generated. Regeneration of TD4-HlyA was done following a first injection of 10 μl of glycine-HCl 10 mM pH 1.7, a second of 5 μl of NaOH 5 mM and a third of 10 μl of glycine-HCl 10 mM pH 1.7. Int280 needed no regeneration. Sensograms with the different concentrations of analyte were overlaid, aligned and analyzed with BIAevaluation 4.1 software (GE Healthcare) under the assumption of the 1:1 Langmuir model and using both the simultaneous kinetics model and the steady-state equilibrium analysis.

10. T3SS-mediated erythrocyte hemolysis

The protocol was performed as previously described[245]. Erythrocytes were obtained from a 5 ml blood sample obtained from rabbits. The blood was treated adding 500 μl of EDTA 1% pH 7.5 (100 μl for each ml of blood) to avoid coagulation and was centrifuged (3500 g, 15 min, RT) to concentrate the erythrocytes. The solution was washed 3 times with one volume of NaCl 0.9% followed by centrifugation (1000 g, 10 min, RT).

The assembly of the T3SS was induced in the indicated cultures as described above until they reached OD_{600} 0.4. Then, 0.5 ml of the cultures and 0.5 ml of the erythrocytes suspension (previously diluted to a 4% in DMEM) were mixed in 1.5 ml tubes. The final mixture was centrifuged (2500 g, 1 min, RT) to induce the contact of the bacteria with the erythrocytes, incubated at 37°C with 5% CO_2 during 4 h, and then the erythrocyte pellets were softly resuspended to homogenize the sample. The samples were centrifuged (12000 g, 1 min, RT) and the hemoglobin liberation to the supernatant was measured at OD_{450} in a spectrophotometer. The hemolysis induced by the wt EPEC strain was considered a 100%, whereas the background hemolysis induced by *E. coli* K-12 strain EcM1 was substracted from the values obtained in all samples. The experiments were done in triplicates.

11. Mammalian cell cultures

The human cell lines HeLa (ATCC, CCL-2) and MDA-MB-231 were grown as monolayer in DMEM, supplemented with 10% fetal bovine serum (FBS) and 2 mM glutamine, at 37°C with 5% CO_2.

11.1. EHEC, EPEC and CR infections

Induced EHEC, EPEC or CR were used for infection of HeLa cell cultures (10^5 cells/well in 24-well tissue culture plates; Falcon) at a multiplicity of infection (MOI) of 300:1 (EPEC) or 1000:1 (EHEC and CR) and 0.1

mM IPTG or 200 nM of purified V_{HH}-HlyA fusions were added at the beginning of the infection, when needed. Infection continued at 37°C with 5% CO_2 for 90 min in the case of EPEC, 150 min for CR and 180 min for EHEC, unless otherwise indicated. The infection was stopped by three washes of sterile PBS. In the case of EHEC infections of 6 h, the cultures were washed 3 times with PBS at 3 h of infection and additional purified V_{HH}-HlyA was added, if needed.

11.2. ß-lactamase assay to determine the translocated proteins

We adapted a previously described method[52]. Briefly, the indicated induced EPEC-derived bacterial strains were used to infect HeLa cells grown *in vitro* in 8-well Falcon culture slides (Beckton Dickinson), 0.1 mM IPTG was added for induction and incubation continued for 90 min. The medium was removed and cells were washed three times with Hank's balance salt solution (HBSS, Sigma). Next, 200 ml of HBSS and 40 ml of the b-lactamase substrate CCF2/AM mix (K1024, Invitrogen) were added. Cells were incubated for additional 90 min at room temperature in the dark, washed three times with HBSS and analyzed by fluorescence microscopy (Olympus BX61, excitation UV light 330–380 nm).

For quantitative analysis of Bla translocation, HeLa cells were seeded in a 96-well opaque plate (Nunc) at aprox. 85% confluence ($2x10^4$ cells/well). After 16 h incubation at 37°C with 5% CO_2, infection were done with pre-activated EPEC strains in serum-free DMEM and the cultures were further incubated for 30 min before addition of IPTG, and 60 min after this addition. Infections were washed three times with HBSS, and 200 ml/well of HBSS were added plus 20 ml of CCF2/ AM substrate mix. Samples were incubated for 90 min in the dark, washed three times with HBSS and finally 100 ml/well of HBSS were added. Plates were read in a SpectraMax M2 fluorometer (Molecular Devices) with a filter set 450/520 nm.

11.3. Immunofluorescence microscopy

Infected HeLa cell cultures, grown on coverslips in 24-well plates, were washed three times with 1 ml/well of PBS, fixed with 4% (w/v) paraformaldehyde (in PBS, 20 min, RT) and washed again with PBS three times. Cells were permeabilized by incubation in a solution of 0.1% (v/v) of saponin (Sigma) in PBS for 10 min. The primary antibodies were added in PBS with 10% goat serum (Sigma) and incubated 60 min at RT. Coverslips were washed three times with PBS. Then coverslips were incubated 45 min with the conjugated secondary antibodies in PBS with 10% goat serum along with Phalloidin TRITC (1:500; Sigma) and DAPI (1:500; Sigma) to label F-actin and DNA, respectively. Coverslips were washed 3 times with PBS after incubation and 4 µl of ProLong Gold anti-fade reagent (Life technologies) was added. They were then observed at the SP5 confocal microscope (Leica) using the 100x objective and an additional 2.5-fold magnification. The list of antibodies used is depicted in Table 8.

11.4. Translocation of Tir with SIEC-eLEE5

Confluent HeLa cells were resuspended from a 75 mm^2 flask in 4 ml of Optimem medium (Life technologies) and 400 µl were electroporated with 10 µl (1 µg/ml) of plasmid pLifeAct-RFP [293] (0.250 kV, 400 µt). The cells were incubated during 20 min, diluted 1:2 in DMEM and 100 µl were seeded in µ-slides 4 well (Ibidi). After 16 h of incubation, the medium was removed, and new complete DMEM medium was added.

SIEC-eLEE5 cultures were transformed with plasmid pGEN22 (Ap^R) for GFP expression. Induced cultures for the expression of the T3SS were incubated with the transfected cells during 90 min using a MOI of 100:1 and then were washed with prewarmed DMEM medium with 0.1 mM IPTG and incubated another 90 min. At that point, the culture was observed at the SP5 microscope (Leica) for 60 seconds time-lapse microscopy during 30 min. The images were processed using the ImageJ software.

12. Purification of injectisomes

The protocol was adapted from previous reports[245]. 20 ml cultures of EPEC and SIEC derived strains were grown in liquid LB o/n with agitation. 19 ml of these cultures were used to inoculate 950 ml of prewarmed DMEM in the case of EPEC or LB in the case of SIEC. Bacteria were grown with gentle agitation (100 rpm) until reaching OD_{600} 0.8 and were then centrifuged to collect the cells (7000 rpm, 15 min). The supernatant was discarded and the pellet was resuspended in 80 ml of sacarose solution (150 mM Tris-HCl pH 8.0, 0.5 M sacarose). The suspension was agitated for homogenization at 4°C and we added 8 ml of freshly prepared 10 mg/ml lysozyme solution drop by drop. Then, we added EDTA pH 8.0 at a final concentration of 2 mM and incubated the solution for 60 min. The cells were then lysed with 0.3% de lauryldimethylamine oxide (LDAO), and we added 12 mM of $MgSO_4$ and 450 mM of NaCl. The color of the solution shifted to be almost transparent. The solution was then centrifuged (13500 rpm, 20 min, 4°C) to take the supernatant. It was then ultracentrifuged (3 h, 42000 rpm, 4°C) and the viscous pellet was resuspended in 2 ml of Buffer F (10 mM Tris-HCl pH 8.0, 0.1% LDAO, 0.3M NaCl, 5mM EDTA). This solution was loaded in a 30% CsCl gradient reaching a final volume of 12 ml, which was ultracentrifuged (40000 rpm, 16 h, 20°C). We collected 500 µl fractions from the gradient and they were diluted with 3.5 ml of buffer F. These fractions were ultracentrifuged (60000 rpm, 30 min, 4°C). The pellets were resuspended in 100 µl of Buffer F and kept on ice for Western blot analysis.

Aliquots of 5 µl from the indicated aliquots containing the injectisomes were applied to collodion-coated copper grids and incubated for 2 min at RT. After that, grids were washed 3 times with MilliQ water to

remove excess of sample and the excess of MilliQ water was removed. For negative staining, samples were incubated with uranyl acetate in a 2% (w/v) solution in MilliQ water for 60 seconds. Excess of uranyl acetate solution was removed and the grids were further washed with MilliQ water. Images were taken with a JEOL 1200EX-II electron microscope operated at 100 kV and recorded at a nominal magnification of 100000X.

For the visualization of the injectisomes on the bacterial surface induced 1ml of cultures of EPEC and SIEC-derived strains were centrifuged softly in 1.5 ml tubes to concentrate the bacteria. 5 µl taken form the bottom of the tube were applied to collodion-coated copper grids and incubated for 2 min at RT. The negative staining was performed as above, but using a 1% (w/v) solution of uranyl acetate and a magnification of 50000X at the electron microscope.

13. Murine model of CR infection

These experiments were performed at the Imperial College in the Gad Frankel laboratory by Valerie Crepin, as previoulsy described[119].

Table 1. Bacterial strains employed in this work

Name	Genotype	Reference
HB2151	K-12 Δ(*lac-proAB*), *ara,* NalR, *thi,* F'[*lacIqZΔM15*, *proAB*]	Carter *et al*., 1985[50]
TG1	Δ(*lac-proAB*) Δ(*mcrB-hsdSM*)5 (*rK- mK-*) *thi-1 supE* [F´ *traD36 proAB lacIqZΔM15*]	Stratagene
BL21 (DE3)	F- *ompT hsdSB*(rB–, mB–) *gal dcm lon* λ(DE3[*lacI lacUV5-T7 gene1 ind1 sam7 nin5*])	Novagen
DH10B-T1R	(F- l-) *mcrA* Δ*mrr-hsdRMS-mcrBC φ80lacZDM15* Δ*lacX74 recA1 endA1 araD139* Δ(*ara, leu*)7697 *galU galK rpsL* (StrR) *nupG tonA*	Novagen
EHEC	EDL933 stx1- stx2- O157:H7	*Perna et al*., 2001[277]
EHECΔ*tir*	EDL933 stx1- stx2- O157:H7 Δ*tir*	DeVinney *et al*., 1999[82]
MG1655	K-12 (F- l-)	Blattner, 1997[31]
EcM1	MG1655, Δ*fimA-H*	Salema *et al*., 2013[308]
EcM1 yeeJ::GFP^{TCD}	EcM1 Δ*yeeJ*::PtacGFP^{TCD}	This work
EcM1 yra::GFP^{TCD}	EcM1 Δ*yra*::PtacGFP^{TCD}	This work
EcM1 yfc::GFP^{TCD}	EcM1 Δ*yfc*::PtacGFP^{TCD}	This work
EcM1 yfaL::GFP^{TCD}	EcM1 Δ*yfaL*::PtacGFP^{TCD}	This work
EcM1 yebT::GFP^{TCD}	EcM1 Δ*yeb*T::PtacGFP^{TCD}	This work
EcM1 flu::GFP^{TCD}	EcM1 Δ*flu*::PtacGFP^{TCD}	This work
EcM1 mat::GFP^{TCD}	EcM1 Δ*mat*::PtacGFP^{TCD}	This work
EcM1 sfm::GFP^{TCD}	EcM1 Δ*sfm*::PtacGFP^{TCD}	This work
EcM1-eLEE2	EcM1 Δ*yeeJ*::PtacLEE2	This work
EcM1-eLEE1*	EcM1 Δ*yfaL*::LEE1	This work
EcM1-eLEE1	EcM1 Δ*yfaL*::PtacLEE1	This work

EcM1-eLEE2,3	EcM1-eLEE2 Δ*yra*::PtacLEE3	This work
EcM1-eLEE2,3,D	EcM1-eLEE2,3 Δ*yfc*::Ptac*escD*	This work
EcM1-eLEE2,3,D,1*	EcM1-eLEE2,3,D Δ*yfaL*::LEE1	This work
SIECΔp1	EcM1-eLEE2,3,D,1* Δ*yebT*::PtacLEE4	This work
SIEC	EcM1-eLEE2,3,D Δ*yfaL*::PtacLEE1 Δ*yebT*::PtacLEE4	This work
EcM1Δ*fliCD*	MG1655Δ*fimA-H* Δ*fliCD*	Majander *et al*., 2005[221]
SIECΔ*flhDC*	SIEC Δ*flhDC*	This work
SIEC-eLEE5	SIEC Δ*flu*::PtacLEE5	This work
SIECΔp1-eLEE5	SIECΔp1 Δ*flu*::PtacLEE5	This work
CC118-λpir	Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA20 thi- rpsE rpoB argE(Am) recA1,* λ*pir*	Herrero *et al*., 1990[136]
BW25141	(F- λ-) Δ*(araD-araB)567*, Δ*lacZ4787*(::rrnB-3), Δ*(phoB-phoR)580*, *galU95*, Δ*uidA3*::*pir*, *recA1*, *endA9*(del-ins)*::FRT*, *rph-1*, Δ*(rhaD-rhaB)568*, *hsdR51*	Datsenko and Wanner, 2000[70]
EPEC	EPEC E2348/69 O127:H6	Iguchi *et al*., 2009[151]
EPECΔ*escN*	E2348/69, Δ*escN*::*aphA3* / KmR	Whale *et al*., 2007[383]
EPECquad	E2348/69, Δ*escF*, Δ*map*, Δ*tir*, Δ*eae* / NalR	Dean *et al*., 2006[74]
ICC169	Spontaneous naladixic acid-resistant variant of CR ICC168	Ghaem-Maghami *et al*., 2001[118]
ICC302	ICC169 Δ*xylE*::Ptet*tccP* / CmR	Girard, 2009[119]
CRΔLEE5	ICC302 ΔLEE5 / ApR	This work
Citro-LEE5$_{EHEC}$	ICC302 ΔLEE5::LEE5EHEC / ApramR	This work

Table 2. Plasmids used in this study

Strain/Plasmid	Relevant genotype and features	Reference	Oligos
pCANTAB5E	Ap^R; pUC ori, for E-tagged fusions of V_{HH}s to the pIII protein of M13	Pharmacia	
pEHlyA4SD	Ap^R; pUC ori, *lac* prom, for E-tagged fusions of V_{HH}s to the HlyA secretion signal	Salema *et al*., 2013[308]	
pEHlyA4SD-IA1	pEHlyA4SD derivative; V_{HH} IA1 clone	This work	
pEHlyA4SD-IB7	pEHlyA4SD derivative; V_{HH} IB7 clone	This work	
pEHlyA4SD-IC1	pEHlyA4SD derivative; V_{HH} IC1 clone	This work	
pEHlyA4SD-IB10	pEHlyA4SD derivative; V_{HH} IB10 clone	This work	
pEHlyA4SD-TF2	pEHlyA4SD derivative; V_{HH} TF2 clone	This work	
pEHlyA4SD-TD4	pEHlyA4SD derivative; V_{HH} TD4 clone	This work	
pEHlyA4SD-TF1	pEHlyA4SD derivative; V_{HH} TF1 clone	This work	
pEHlyA4SD-TG10	pEHlyA4SD derivative; V_{HH} TG10 clone	This work	
pEHlyA4SD-TE1	pEHlyA4SD derivative; V_{HH} TE1 clone	This work	
pEHlyA4SD-TE4	pEHlyA4SD derivative; V_{HH} TE4 clone	This work	
pEHlyA4SD-EE6	pEHlyA4SD derivative; V_{HH} EE6 clone	This work	
pEHlyA4SD-EC7	pEHlyA4SD derivative; V_{HH} EC7 clone	This work	
pEHlyA4SD-EC1	pEHlyA4SD derivative; V_{HH} EC1 clone	This work	
pEHlyA5-TD4	pEHlyA4SD derivative; N-terminal His-tagged V_{HH} TD4 fused to the HA- and E-tagged HlyA signal	This work	
pEHlyA5-IB10	pEHlyA5-TD4 derivative; V_{HH} IB10 clone	This work	
pEHlyA5-EC7	pEHlyA5-TD4 derivative; V_{HH} EC7 clone	This work	

pEHlyA5-Vamy	pEHlyA4SD derivative; Vamy clone	This work	
pVDL9.3	Cm^R; pSC101 ori, *lac* prom, production of HlyB and HlyD transporters	Fernández *et al*., 2000[100]	
pVDL9.3-TD4	pVDL9.3 derivative; operon expressing TD4-HlyA,HlyB and HlyD	This work	
pVDL9.3-Vamy	pVDL9.3 derivative; operon expressing Vamy-HlyA,HlyB and HlyD	This work	
pSA10	Ap^R; pBR ori, *lac* prom and lacIq	Schlosser-Silverman *et al*., 2000[317]	
pT3sVgfp	pSA10 derivative; EspF secretion signal fused to His- and E-tagged Vgfp	Blanco-Toribio *et al*., 2010[29]	
pT3sVcapG2	pT3sVgfp derivative; VcapG2 clone	This work	
pT3sVcapG4	pT3sVgfp derivative; VcapG4 clone	This work	
pCX340	Tc^R; pBR ori, *trc* prom, for fusions to the ß-lactamase gene	Charpentier and Oswald, 2004[52]	
pT3s-bla	pCX340 derivative; EspF secretion signal fused to the ß-lactamase	Blanco-Toribio *et al*., 2010[29]	
pT3sVgfp-bla	pT3s-bla derivative; Vgfp clone	Blanco-Toribio *et al*., 2010[29]	
pT3sVcapG2-bla	pT3s-bla derivative; VcapG2 clone	This work	
pT3sVcapG4-bla	pT3s-bla derivative; VcapG4 clone	This work	
pET28a	Km^R; pBR ori, T7 prom, for N-terminal His-tagged fusions	Novogen	
pET28a-TirM$_{EHEC}$	pET28a derivative; TirM sequence of EHEC	Salema *et al*., 2013[308]	5, 6
pET28a-Int280$_{EHEC}$	pET28a derivative; Int280 sequence of EHEC	This work	7, 8
pET28a-EspA$_{EHEC}$	pET28a derivative; EspA sequence of EHEC	This work	9, 10
pET28a-TirM$_{EPEC}$	pET28a derivative; TirM sequence of EPEC	This work	11, 12
pET28a-TirM$_{CR}$	pET28a derivative; TirM sequence of *C. rodentium*	This work	13, 14
pACBSR	Cm^R; p15A ori, BAD prom, I-SceI endonuclease and λ Red genes	Herring *et al*., 2003[137]	
pACBSR	Sp^R; p15A ori, BAD prom, I-SceI endonuclease and λ Red genes	Cepeda M. (unpublished)	
pCP20	Ap^R and Cm^R; pSC101-ts prom, expression of the flippase gene at 37°C	Cherepanov and Wackernagel, 1995[54]	
pGEflu	Km^R; R6K ori, HRs to delete the *flu* gene flanked by I-SceI restriction sites	Piñero-Lambea *et al*., 2014[279]	
pGEfluPtacGFP^{TCD}	pGEflu derivative; *tac* prom and GFP^{TCD} between the *flu* HRs	This work	15, 16
pGEyeeJPtacGFP^{TCD}	pGEfluPtacGFP^{TCD} derivative; *yeeJ* HRs	This work	17, 18, 19, 20
pGEyraPtacGFP^{TCD}	pGEfluPtacGFP^{TCD} derivative; *yra* HRs	This work	27, 28, 29, 30
pGEyfcPtacGFP^{TCD}	pGEfluPtacGFP^{TCD} derivative; *yfc* HRs	This work	37, 38, 39, 40
pGEyfaLPtacGFP^{TCD}	pGEfluPtacGFP^{TCD} derivative; *yfaL* HRs	This work	47, 48, 49, 50
pGEyebTPtacGFP^{TCD}	pGEfluPtacGFP^{TCD} derivative; *yebT* HRs	This work	64, 65, 66, 67
pGEsfmPtacGFP^{TCD}	pGEfluPtacGFP^{TCD} derivative; *sfm* HRs	This work	74, 75, 76, 77
pGEmatPtacGFP^{TCD}	pGEfluPtacGFP^{TCD} derivative; *mat* HRs	This work	80, 81, 82, 83
pGETS	Km^R; pSC101-ts ori, polilynker flanked by I-SceI sites	Cepeda M. (unpublished)	
pGETSyeeJPtacLEE2	pGETS derivative; *tac* prom and EPEC eLEE2 sequence between by the *yeeJ* HRs	This work	21, 22
pGETSyraPtacLEE3	pGETS derivative; *tac* prom, EPEC eLEE3 sequence	This work	31, 32, 92, 93

	and Ap^R (flanked by FRTs) between *yra* HRs		
pGETSyfcPtacEscD	pGETS derivative; *tac* prom, EPEC *escD* sequence and Ap^R (flanked by FRTs) between *yfc* HRs	This work	41, 42, 92, 93
pGETSyfaLLEE1*	pGETS derivative; EPEC eLEE1 sequence and Ap^R (flanked by FRTs) between *yfaL* HRs	This work	51, 52, 53, 54, 55, 56, 94, 95
pGEyfaLPtacEscE	pGEyfaLPtacGFP^{TCD} derivative; *tac* prom between *yfaL* and EPEC *escE* HRs	This work	57, 58
pGETSyebTPtacLEE4	pGETS derivative; *tac* prom, EPEC eLEE4 sequence and Ap^R (flanked by FRTs) between *yebT* HRs	This work	68, 69, 92, 93
pGETSfluPtacLEE5EPEC	pGETS derivative; *tac* prom, EPEC eLEE5 sequence and Ap^R (flanked by FRTs) between *flu* HRs	This work	86, 87, 92, 93
pGEΔflhDC	pGEflu derivative; HRs to delete the *flhDC* operon	This work	96, 97, 98, 99
pGETSΔLEE5CR	pGETS derivative; Ap^R (flanked by FRTs) between HRs to delete the LEE5 operon of *C. rodentium*	This work	102, 103, 104, 105, 106, 107
pGETSLEE5EHEC	pGETSΔLEE5CR derivative; the native LEE5 operon of EHEC and $Apram^R$ between the HRs	This work	108, 109, 110, 111
pCVD462	Cm^R; p15a ori, harbours the LEE island of EPEC	McDaniel and Kaper, 1997[234]	
pLifeAct-RFP	Ap^R; chicken beta-actin promoter, for actin staining *in vivo*	Riedl *et al*., 2010[294]	
pGEN22	Ap^R; ori p15a, stable expression of GFP^{UV}	Galen *et al*., 1999[110]	

Table 3. Oligonucleotides used in this study

Number	Name	Sequence (5'-3')
1	CALL001	GTCCTGGCTCTCTTCTACAAGG
2	CALL002	GGTACGTGCTGTTGAACTGTTCC
3	V_{HH}-SfiI2	GTCCTCGCAACTGCGGCCCAGCCGGCCATGGCTCAGGTGCAGCTGGTGGA
4	V_{HH}-NotI2	GGACTAGTGCGGCCGCTGAGGAGACGGTGACCTGGGT
5	BamEcoTirM-EHEC	CGCGGATCCGAATTCCAGGCGCTTGCATTGACGCCGGAG
6	XhoHindTirM-EHEC	CCGCTCGAGAAGCTTTTACGATGAAACTTTCAGCTCCTCCTG
7	BamEcoInt280-EHEC	CGCGGATCCGAATTCATTACTGAGATTAAGGCTGATAAG
8	XhoHindInt280-EHEC	CCGCTCGAGAAGCTTTTATTCTACACAAACCGCATAGAC
9	BamEcoEspA-EHEC	CGCGGATCCGAATTCATGGATACATCAAATGCAACATCC
10	XhoHindEspA-EHEC	CCGCTCGAGAAGCTTTTATTTACCAAGGGATATTGCTGA
11	BamEcoTirM-EPEC	CGCGGATCCGAATTCCAGGCGTTGGCTTTGACACCGG
12	XhoHindTirM-EPEC	CCGCTCGAGAAGCTTACCCGATGAAAGCTGTAATTCCTCCTG
13	BamEcoTirM-CR	CGCGGATCCGAATTCCAGGCGGTTGCTTTGACACCAGC
14	XhoHindTirM-CR	CCGCTCGAGAAGCTTTATGATGAGAGATCCAATTCCTGCCGC
15	5' gfpTCD	GATAAGCTTTAGGGCCCATGCTAGCTTTGCGGCCGCAACCTAGGAAGGATCCAAGAAGGAGATAACATATG
16	3' gfpTCD	GAATTCACTAGTTTACTTATACAGTTCATCCA
17	yeeJ HR UP 5' XhoI	GCTTCTCGAGGGCGATCGCGCCAGCGGAGT
18	yeeJ HR UP 3' SacI	CGTAGAGCTCGCGAATTTTTAGAATTTGGC
19	yeeJ HR DO 5' SpeI	GCTTACTAGTTTTTCCGTTATAATTTCTTAAAGAG
20	yeeJ HR DO 3' SphI	GATTGCATGCCGATTAAACGTTCAGCAGCA
21	5' HindIII LEE2 EPEC	CGTATAAGCTTAAGAAGGAGATATACATATGGATGCATTATGCTATTGC
22	3' SpeI LEE2 EPEC	GATACACTAGTTTACTCTGTATTACCTAAC

23	Integra UP yeeJ	ACCTGATTATTCATGAAGTCGCTCATAAGC
24	Integra DO yeeJ	AAGGTGAAGATAAAGCCAGGGCATTAGTGG
25	3’ LEE2 integra UP	ATAACAACACTAATATTTTC
26	5’ LEE2 integra DO	GAGTTCGTTTGTTACTTGATATTC
27	yra HR UP 5’ XhoI	GCTTCTCGAGCTTGGGCTGAACGAACCGGG
28	yra HR UP 3’ SacI	GGTCGAGCTCAGAGTATAAATGCCTGACTTTG
29	yra HR DO 5’ SpeI	GCATACTAGTCATGGCACCGTCAATATGCG
30	yra HR DO 3’ SphI	GATTGCATGCGAATCTACCCACCGTCTGTTAG
31	5’ SalI LEE3 EPEC new	CGTATGTCGACAAGAAGGAGATATACATATACATATGAATCTTTTAGTTAAAAGAAATGTTGAAG
32	3’ SpeI LEE3 EPEC	GATACACTAGTTTAATCACATACTACGCTAATAG
33	Integra UP yra	GCGGTCTGGATTTATGCGTTCTCGGATTGG
34	Integra DO yra	TTTTTACCTGCCAAATCAAAAGGCC
35	3’ LEE3 integra UP	GCAGCTCTTTCACAAGCTCG
36	5’ LEE3 integra DO	CGATGGGGCAAGATCCTGAAG
37	yfc HR UP 5’ XhoI	GCTTCTCGAGGGCAGCGGATATCTGTCAAC
38	yfc HR UP 3’ SacI	GGTCGAGCTCGTATTCGTTGGTGTGGGGTC
39	yfc HR DO 5’ SpeI	GCATACTAGTGGACAAACAGTTACACGATATG
40	yfc HR DO 3’ SphI	GATTGCATGCGTTCGCCCGGATGTCAGCC
41	5’ SalI EscD EPEC	GTCATGTCGACAAGAAGGAGATATACATATGTTATCCTCATATAAAATAAAAC
42	3’ SpeI EscD EPEC	GATACACTAGTTTAATACGACAGTGGAATATG
43	Integra UP yfc	CAATGAGGTAGATTCATTGGGCATAATTGC
44	Integra DO yfc	CAGCCGTTATTAAATACCGAAGGTCCGGTG
45	3’ EscD integra UP	CTTATCCTGGCGAGCGTGATTGGTGC
46	5’ EscD integra DO	AATGGCAAGGATCTTACTCTCGTTCTG
47	yfaL HR UP 5’ XhoI	GCTTCTCGAGCCTGTAGTACGGGAAGCTGC
48	yfaL HR UP 3’ SacI	GGTCGAGCTCATAACCTTCAGGGATATCAG
49	yfaL HR DO 5’ SpeI	GCATACTAGTCAGTCAGCGAGTGTCCCTGC
50	yfaL HR DO 3’ SphI	GATTGCATGCCAACTGGTGAAACCAGGCGG
51	5’ HindIII pGE+XmaI,NcoI	TCGACCTGCAGGTTTGCAAGC
52	3’ SpeI pGE+Xma,NcoI	TGAGACTAGTCCATGGCCCGGGTTACTTATACAGTTCATCCATACC
53	5’ SacI pGETS-prom Xba	CCAGGGCTACACTAGTCGTTGAGGTT
54	3’ SacI pGETS-prom Xba	TCTAGAACCTCAACGACTAGTGTAGCCCTGGAGCTC
55	5’ SalI LEE1 EPEC	CGTATGTCGACAAGAAGGAGATATACATATGCGGAGATTATTTATTATG
56	3’ NcoI,XmaI LEE1 EPEC	GATACCCATGGCCCGGGTTAATAATCAAGGTCTATCGC
57	5’ Sac,Sal,Avr LEE1 EPEC-ler	CGTATGAGCTCGTCGACCCTAGGAAGAAGGAGATATACATATCATAACGATAACTGAGCTGGAAGACG
58	3’ Sph HR escE CesAB	CTTGGTGCATGCTCATACTATTTTTCTATTATTTCTATTCCG
59	Integra UP yfaL	TGGAATTCTTTCCGCAATAGTTTCATGCAG
60	Integra DO yfaL	TGGTGACCTGCATGGAGATGCTGGAGCACG
61	3’ LEE1 integra UP	CAATCTTACTTAGGGCAGAATC
62	5’ LEE1 integra DO	TTGCTAATTTCACAGGACTG
63	3’ PtacEscE integra	TCGAATCCTTTCTCTCATTGC
64	yebT HR UP 5’ XhoI	GCTTCTCGAGCGCTGTTAGCGTCAATCG
65	yebT HR UP 3’ SacI	GGTCGAGCTCCGTTTATTTTTAATCTGCGC
66	yebT HR DO 5’ SpeI	GCATACTAGTGGTACGCCACTGGCACCGAAAGC

67	yebT HR DO 3’ SphI	GATTGCATGCGCAACATTGAACTGGCTTCC
68	5’ SalI LEE4 EPEC	CGTATGTCGACAAGAAGGAGATATACATATGGCTAATGGTATTGAATTTAATC
69	3’ SpeI LEE4 EPEC	GATACACTAGTTTAAAATCCTCGTACCCAACC
70	Integra UP yebT	CTGCGTCGCCGTCATAGTCTGCAAAAATGC
71	Integra DO yebT	GCTGTGGTGCATTACCGTCAGCAAACAAGG
72	3’ LEE4 integra UP	CAAGCATCAGGTAATGATGC
73	5’ LEE4 integra DO	GTTACCAGCCTTGCTGAAGG
74	sfm HR UP 5’ XhoI	GCTTCTCGAGGTTACTACCCACCAGCACAAC
75	sfm HR UP 3’ SacI	GGTCGAGCTCGTCTTAATTGAAAATATGCAC
76	sfm HR DO 5’ SpeI	GCATACTAGTGTCACACTAAACTTTTTTGC
77	sfm HR DO 3’ SphI	GATTGCATGCGAAAGTGTTATTTTTATCATC
78	Integra UP sfm	TTCTTCACAAGCCTTGCGTTTGCTTGCGAC
79	Integra DO sfm	TTACCTTCCTGAAAAGGATCAAACAAATCC
80	mat HR UP 5’ XhoI	CCGCTCGAGCTGAACTGATTGTGGATATCGACAG
81	mat HR UP 3’ SacI	CCGGAGCTCTGCATTTCTTCCCGAGTTGAATTGAGG
82	mat HR DO 5’ SpeI	GACTAGTGCATCTGGAGCGGCGACGTTAGCGTAC
83	mat HR DO 3’ SphI	ACATGCATGCCACAGCGCTGCGGTTGGCATTATCG
84	Integra UP mat	ACTCAGTCTCCTCCCTTTGCG
85	Integra DO mat	ACTTATGTCAGCAGCGCTGGC
86	5’ HindIII LEE5 EPEC	AGCTAAGCTTAAGAAGGAGATATACATATGCCTATTGGTAACCTTGG
87	3’ SpeI LEE5 EPEC	GACTACTAGTTTATTTTACACAAGTGGCATAAGC
88	Integra UP flu	CGGTTCACAGGCAATTGGCGGTATTGTTAAC
89	Integra DO flu	ACATCAGTGACGGTGAAATATCGTACTGTAACG
90	3’ LEE5 EPEC integra UP	CAGCCTCTTTAGCTTGTTGTGC
91	5’ LEE5 EPEC integra DO	TGCCAAAGTAACATTAACATCG
92	F.SacI.SpeI.Amp.FRT	CGCGAGCTCACTAGTGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCGGAATAGGAACTTCATGAGTAAACTTGGTCTGAC
93	R.SpeI.Amp.FRT	ATACTAGTGAAGTTCCTATTCCGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTCTCGGGGAAATGTGCGCGGAACC
94	F.SacI.NcoI.Amp.FRT	CGCGAGCTCCCATGGGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCGGAATAGGAACTTCATGAGTAAACTTGGTCTGAC
95	R.SacI.NcoI.Amp.FRT	CGCGAGCTCCCATGGGAAGTTCCTATTCCGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTCTCGGGGAAATGTGCGCGGAACC
96	5’ XhoI HR UP flhDC	GATCCTCGAGCATTTATGTTAAGTAATTGAGTGTTTTG
97	3’ SacI HR UP flhDC	GATCGAGCTCTATTCCCACCCAGAATAACCAAC
98	5’ SacI HR DO flhDC	GATCGAGCTCCTGATACGGTGAGGCGCAACATTCC
99	3’ SphI HR DO flhDC	GATCGCATGCTACATTGCTTTGGTGTATTTGGAGC
100	Integra UP flhDC	AGTTATTTTGACTGTGCGCAACATCC
101	Integra DO flhDC	CGATTTCGCCATCAACCGATAAAGC
102	LEE5 Cit HR UP 5’ XhoI	GCTTCTCGAGGGAGTGGATCCTATTACACG
103	LEE5 Cit HR UP 3’ XbaI	GGTCTCTAGAACATATATCCTTTTATTTAATCGG
104	LEE5 Cit HR DO 5’ HindIII	GCATAAGCTTTGATATTTTATTTAACATATCTATAGCATCTACACAATAAAAAAGATTCGAAGAGG
105	LEE5 Cit HR DO 3 SphI	GATTGCATGCGGGATTCTTCCTGCTATCGGG
106	F.HindIII.Amp.FRT	CTAAAGCTTGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCGGAATAGGAACTTCATGAGTAAACTTGGTCTGACAG
107	R.SacI.HindIII.Amp.FRT	CGCGAGCTCAAGCTTGAAGTTCCTATTCCGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTCTCGGGGAAATGTGCGCGGAACC

108	LEE5 EHEC 5' Xba+RBS	GTTCTCTAGAAAAGGAGATATTTATGCCTATTGG
109	LEE5 EHEC 3' HindIII	GCATAAGCTTTTATTCTACACAAACCGCATATAGAC
110	F.HindIII.Apram.FRT	CAGTAAGCTTGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCGGAATAG GAACTTCGGAATAGGAACTTATGAGCTCAGCC
111	R.HindIII.Apram.FRT	CAGTAAGCTTGAAGTTCCTATTCCGAAGTTCCTATTCTCTAGAAAGTATAG GAACTTCTGCAGCTCACGGTAACTGATGCC
112	Integra UP LEE5 Cit	GCAGCCTCTTGATATAAATTGTGCACAATCATC
113	Integra DO LEE5 Cit	GTATGGCATGGGAAAAACGTCACACTTTATGGACG
114	5' LEE5 EHEC no Cit	CTCTGTTGTTTTAAGTGATCAAGAGTACGCTC
115	3' LEE5 EHEC no Cit	ATTTGCAGGCTTATTCTCTACCGTACGTGCGCTTG

Table 4. Modifications of the genome using pGE plasmids

Strain to be modified	pGE plasmid	Resulting strain	Oligos to check the integration
EcM1	pGEyeeJPtacGFP^{TCD}	EcM1 yeeJ::GFP^{TCD}	23, 24
EcM1	pGEyraPtacGFP^{TCD}	EcM1 yra:: GFP^{TCD}	33, 34
EcM1	pGEyfcPtacGFP^{TCD}	EcM1 yfc:: GFP^{TCD}	43, 44
EcM1	pGEyfaLPtacGFP^{TCD}	EcM1 yfaL:: GFP^{TCD}	59, 60
EcM1	pGEyebTPtacGFP^{TCD}	EcM1 yebT:: GFP^{TCD}	70, 71
EcM1	pGEsfmPtacGFP^{TCD}	EcM1 sfm:: GFP^{TCD}	78, 79
EcM1	pGEmatPtacGFP^{TCD}	EcM1 mat:: GFP^{TCD}	84, 85
EcM1	pGEfluPtacGFP^{TCD}	EcM1 flu:: GFP^{TCD}	88, 89
SIEC	pGEΔflhDC	SIECΔflhDC	100, 101
EcM1-eLEE1*	pGEyfaLPtacEscE	EcM1-eLEE1	59, 63
SIECΔp1	pGEyfaLPtacEscE	SIEC	59, 63

Table 5. Modifications of the genome using pGETS plasmids

Strain to be modified	pGETS plasmid	Resulting strain	Oligos to check the integration
EcM1	pGETSyeeJPtacLEE2	EcM1-eLEE2	23, 24, 25, 26
EcM1-eLEE2	pGETSyraPtacLEE3	EcM1-eLEE2,3	33, 34, 35, 36
EcM1-eLEE2,3	pGETSyfcPtacEscD	EcM1-eLEE2,3,D	43, 44, 45, 46
EcM1-eLEE2,3,D	pGETSyfaLLEE1*	EcM1-eLEE2,3,D,1*	59, 60, 61, 62
EcM1-eLEE2,3,D,1*	pGETSyebTPtacLEE4	SIECΔp1	70, 71, 72, 73
EcM1	pGETSyfaLLEE1*	EcM1-eLEE1*	59, 60, 61, 62
SIEC	pGETSfluPtacLEE5EPEC	SIEC-eLEE5	88, 89, 90, 91
SIECΔp1	pGETSfluPtacLEE5EPEC	SIECΔp1-eLEE5	88, 89, 90, 91
ICC302	pGETSΔLEE5CR	CRΔLEE5	112, 113
CRΔLEE5	pGETSLEE5EHEC	Citro-LEE5$_{EHEC}$	112, 113, 114, 115

Table 6. Antibodies used for Western blot		
Protein	**Primary antibody**	**Secondary antibody**
V_{HH}-HlyA / T3s-V_{HH}	anti-E-tag (Phadia, 1:5000)	anti-mouse IgG-POD (1:5000; Sigma)
V_{HH}-HlyA	anti-HA-tag	anti-mouse IgG-POD (1:5000; Sigma)
GroEL	anti-GroEL mAb-POD (Sigma, 1:5000)	---
T3s-V_{HH}-bla	anti-ß-lactamase mAb (QED Bioscience, 1:1000)	anti-mouse IgG-POD (1:5000; Sigma)
EscC	anti-EscC (1:1000)	Protein A-HRP (Life Technologies, 1:5000)
EscD	anti-EscD (1:1000)	Protein A-HRP (Life Technologies, 1:5000)
EscN	anti-EscN (1:500)	Protein A-HRP (Life Technologies, 1:5000)
EspA	anti-EspA (1:2000)	Protein A-HRP (Life Technologies, 1:5000)
EspB	anti-EspB (1:2000)	Protein A-HRP (Life Technologies, 1:5000)
EscJ	anti-EscJ (1:5000)	Protein A-HRP (Life Technologies, 1:5000)
Tir_{EPEC}	anti-Tir_{EPEC} (1:5000)	Protein A-HRP (Life Technologies, 1:5000)
$Int280_{EPEC}$	anti-$Int280_{EPEC}$ (1:5000)	Protein A-HRP (Life Technologies, 1:5000)

Table 7. Peptides used for the generation of rabbit antibodies	
Protein	**Sequence**
EscN	STKDISSYEKTIESLFKVVA
EscD	CNISRPDEYIHIPLSY
EscC	CVVRLFLIKATPIKSASSE

Table 8. Substrates used for fluorescence and confocal microscopy		
Antigen	**Substrate for detection**	**Secondary antibody**
EHEC	Mouse mAb anti-O157 (Abcam, 1:500)	Goat anti-mouse-ALEXA488 (Life technologies)
Tir_{EHEC}	Polyclonal rabbit Ab anti Tir of EHEC (1:200)	Goat anti-rabbit-ALEXA647 (Life technologies)
EPEC	Polyclonal rabbit Ab anti-intimin (1:500)	Goat anti-rabbit-ALEXA488 (Life technologies)
CR	Polyclonal rabbit Ab anti-CR (1:100)	Goat anti-rabbit-ALEXA488 (Life technologies)
Actin	Phalloidin-TRITC/Phalloidin-ALEXA488 (1:500)	
DNA	DAPI (4',6-diamidino-2-phenilindole, 1:500, Life technologies)	

RESULTS

Chapter 1: Inhibition of the infection of EHEC with a secreted anti-Tir antibody

1. Screening of V_{HH} antibodies against TirM, Int280 and EspA

1.1. Generation of enriched immune libraries against TirM, Int280 and EspA of EHEC

In this project, we target key proteins - Int, Tir and EspA - that participate in the formation of the EHEC-derived A/E lesion with V_{HH} antibodies, to test whether they interfere with the intimate attachment of EHEC to the host cells. As a starting point, we purified His-tagged versions of EHEC EspA protein and of the protein domains involved in the Int:Tir interaction: Int280, the carboxi-terminal 280 aminoacids of Int, and TirM, the extracellular domain (aminoacids 252 to 360) of Tir. These polypeptides were overproduced and purified from the cytoplasm of *E. coli* BL21(DE3) (Figure 11A) and used for the immunisation of a dromedary camel (see materials and methods). We tested the humoral immune response against these antigens in the camel blood serum using ELISA plates coated with Int280, TirM and EspA and the control protein BSA, detecting a specific and strong immune response of the camel against each of these antigens (Figure 11B). The strongest response was obtained against EspA, followed by Int280 and TirM, which showed similar behavior.

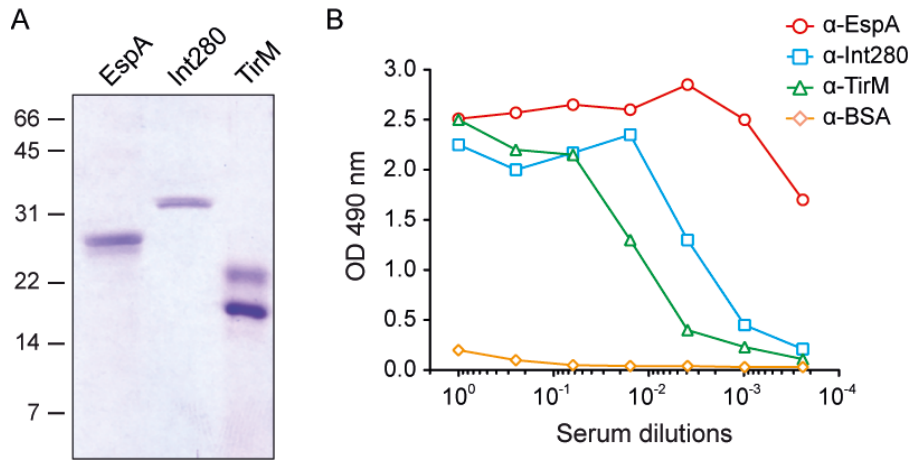


Figure 11. Camel immunisation with purified EspA, Int280 and TirM. A. Coomassie staining of the proteins that were injected to a camel in 5 independent immunisations. B. ELISA of the camel serum after immunisation against each of the injected proteins and the control antigen BSA. The immune response was assayed against each of the proteins using serum dilutions.

We isolated the lymphocytes from a blood sample of the immunized animal (ca. $2x10^7$ cells), prepared mRNA and and amplified the V_{HH} genes by RT-PCR following standard procedures. The V_{HH} genes were cloned in a phagemid vector (pCANTAB5E) and transformed into *E. coli* strain TG1 to generate a phage antibody (Phab) library with a diversity of 10^7 clones. By infecting the library with a helper phage (VCS-M13) we obtained the filamenteous phage particles displaying the V_{HH} proteins and the rescued Phabs were panned by incubation with ELISA plates coated with TirM, Int280 or EspA. Phabs bound to each antigen

were recovered by acidic elution (0.1 M Glycine, pH 2.5) and used to infect *E. coli* TG1 and generate independent sublibraries for each antigen. Each sublibrary was grown, rescued as Phabs and we subjected it to a second round of panning with the respective antigen for further enrichment of antigen-binders (Figure 12).

1.2. Selection of V_{HH} clones binding TirM, Int280 and EspA using the *E. coli* HlyA secretion system.

In order to identify V_{HH} clones binding TirM, Int280 or EspA that could be secreted through the HlyA system, the pool of V_{HH} genes from the enriched Phab sublibraries were cloned in the pEHLYA4SD vector, thus generating three independent libraries of V_{HH}s fused to the C-terminal secretion signal of HlyA. The V_{HH}-HlyA libraries were then transformed into the *E. coli* strain HB2151 (TolC+) carrying plasmid pVDL9.3 (HlyB+, HlyD+) to allow secretion. We cultured 96 individual clones from each library in liquid medium and induced them with IPTG to secrete the V_{HH}-HlyA fusions to the extracellular medium. We isolated the supernatants of 96 cultures from each library parting from individual colonies to detect those recognising Int280, TirM and EspA in ELISA, using BSA as a control antigen (Figure 12). Given that the libraries were previously enriched, we found that 40-80% of the screened clones had a positive signal against their corresponding antigen and not against BSA.

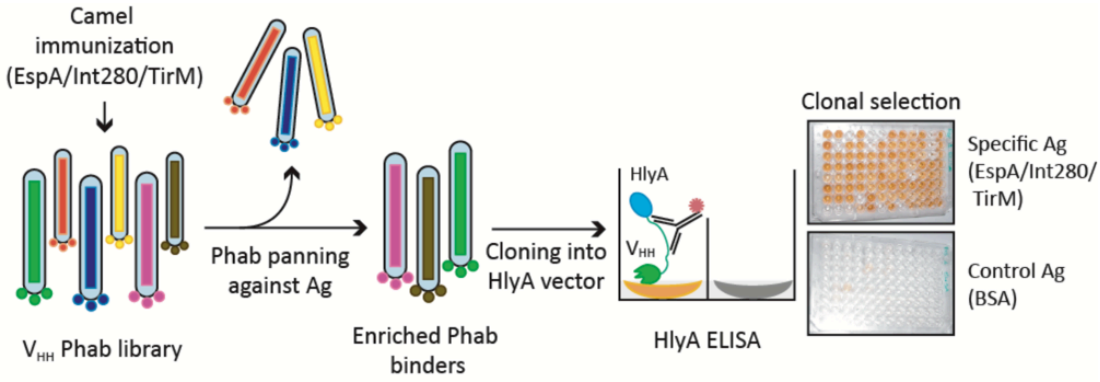


Figure 12. Selection of V_{HH} antibodies from the camel immune library. The V_{HH} sequences of the heavy chain only antibodies were amplified by PCR and cloned in plasmid pCANTAB5E to generate a phage antibody (Phab) library. The library was enriched by panning against each of the antigens and sublibraries were cloned into the HlyA vector pEHlyA4SD. The supernatants of individual clones from each library were tested against their respective antigens and the control antigen BSA in ELISA to detect positive and specific binders.

We selected from each sublibrary a set of 24 to 40 clones with high binding signals in the ELISA screening. The primary sequences of these V_{HH}s were determined by DNA sequencing and aligned to identify those with different complementarity determining regions (CDRs). This analysis revealed that most of the clones can be sorted into independent groups of related sequences. We found four groups of sequences coding V_{HH}s against Int280 (A1, B7, B10, C1), six groups in the case of V_{HH}s against TirM (D4, E1, E4, F1, F2 and G10), and three for V_{HH}s against EspA (C1, C7, E6) and selected a representative V_{HH} clone for each group. Table 9 summarizes the identified V_{HH} groups, their frequency of isolation, if they were also identified by a

Phage display screening performed in parallel and the primary sequence of the CDR3 of from each group. These clones were named after their recognising antigen (E for EspA, T for TirM and I for Int280).

Table 9. Summary of identified Nanobodies				
Antigen	Representative clone	Frequency	Identified by phage display	CDR3 sequence
Int280	IA1	14/27	Yes	GIYYSV--------FSVCAGRIEH
Int280	IB7	6/27	No	DSGPY------CLDCGYC-DRYNY
Int280	IC1	6/27	Yes	RQGVTS---------WLRDTEYSY
Int280	IB10	1/27	Yes	ELGAG----------SGRCYGYHY
TirM	TF2	10/24	Yes	PKYGGT------WRWRVEEEKTTI
TirM	TD4	4/24	No	SAGHTIRTVTSC-----PKYGINY
TirM	TF1	4/24	No	PDLSTN----CDTVLTNSGALYNY
TirM	TG10	4/24	Yes	GTAPYW----HTPIPTLSEDKYFY
TirM	TE1	1/24	No	DRCHSSTQVAGFGTNPRGRYGYAY
TirM	TE4	1/24	No	DRRVHF------CKAPLSTSGHDT
EspA	EC7	33/40	Yes	ATDSYLCNPS--------RGGYNV
EspA	EC1	6/40	Yes	G---------GGRLGWGAMASFAY
EspA	EE6	1/40	No	GNQYSD-----GGCRYSGTRGYNN

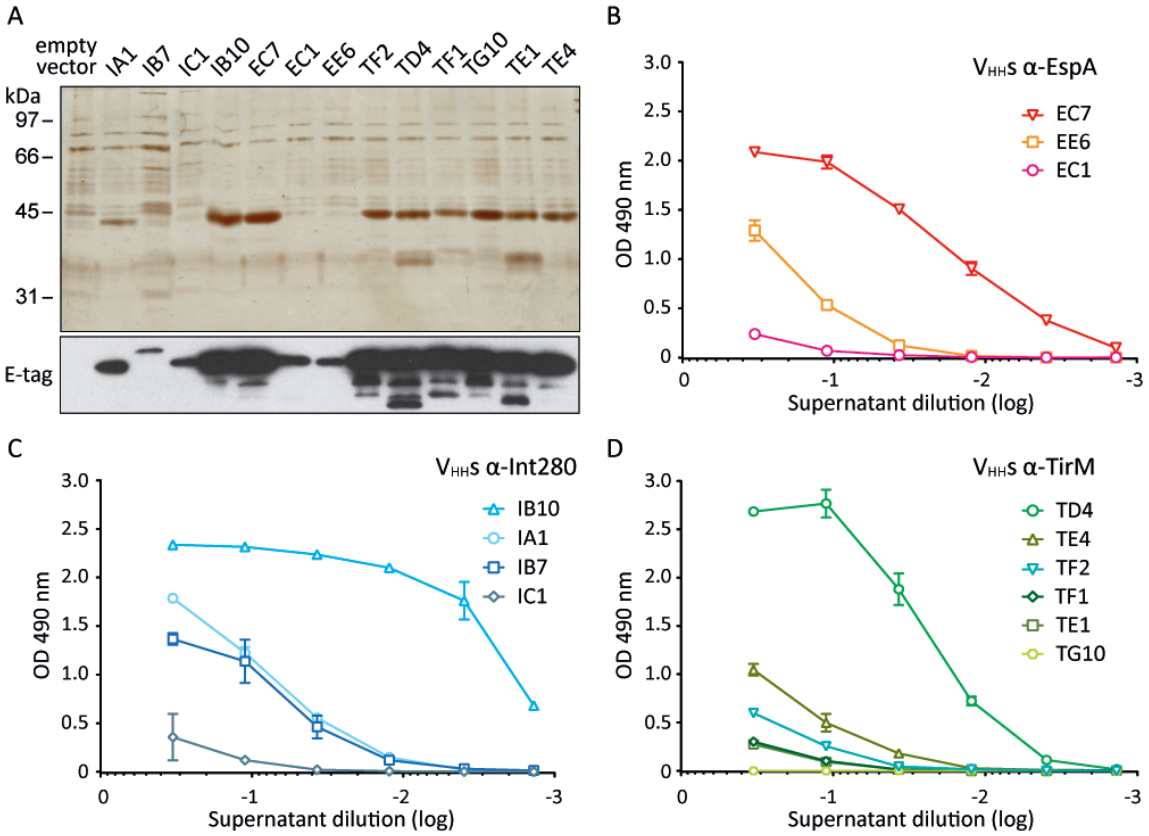


Figure 13. Secretion yields and relative affinity of the representative V_{HH} clones. The representative clones of the identified groups were subjected to secretion by the HlyA system. A. Silver staining of the supernatant obtained from the induced cultures of each clone showing the secretion yields obtained of the 45 kDa V_{HH}-HlyA fusions. The clones were also identified by E-tag detection in Western blot. In B, C and D is shown the relative affinity determination of the supernatants of the anti-EspA, anti-Int280 and anti-TirM clones, respectively, done by supernatant 3-fold dilutions and in triplicates.

Subsequently, we wanted to elucidate the secretion levels of the V_{HH}-HlyA secreted clones. Thus, we cultured the representative clones of each group and induced the secretion to analyze the corresponding supernatants by silver staining and Western Blot. We used the pEHLYA4-SD empty vector as a negative control. All of the selected V_{HH} fusions were detected in the culture supernatant at the expected size (45 kDa). The secretion of most of the clones was clearly detectable by silver staining, though we observed differences in the secretion level among them (Figure 13). We also compared the binding affinities of the V_{HH}s by testing serial dilutions of the culture supernatants in ELISA. Dilutions of culture supernatants were analyzed by ELISA revealing important differences in their binding signals against the antigens. These data allowed us to select clones TD4, IB10 and EC7, given their high binding signals and good secretion levels.

2. Inhibition of the intimate attachment of EHEC *in vitro*

2.1. Inhibitory capacity of the selected V_{HH}s

We then cloned the selected antibodies in pEHLYA5, harbouring a His-tag at the N-terminal for purification, and HA-tag and E-tag for detection. We transformed *E. coli* strain HB2151 with pVDL9.3 and the respective pEHLYA5 plasmids, and induced LB liquid cultures with IPTG for the secretion of the V_{HH}-HlyA fusions. The supernatants were collected by centrifugation and the antibodies were purified. We quantified the production yields to be between 1-4 mg/L of culture. Next, we tested their apparent affinity in ELISA. Starting at a concentration of 1 µM, we made 3-fold dilutions and observed that all of them showed high affinity for their respective antigens, with apparent affinities (K_Ds) in the nM range (Figure 14).

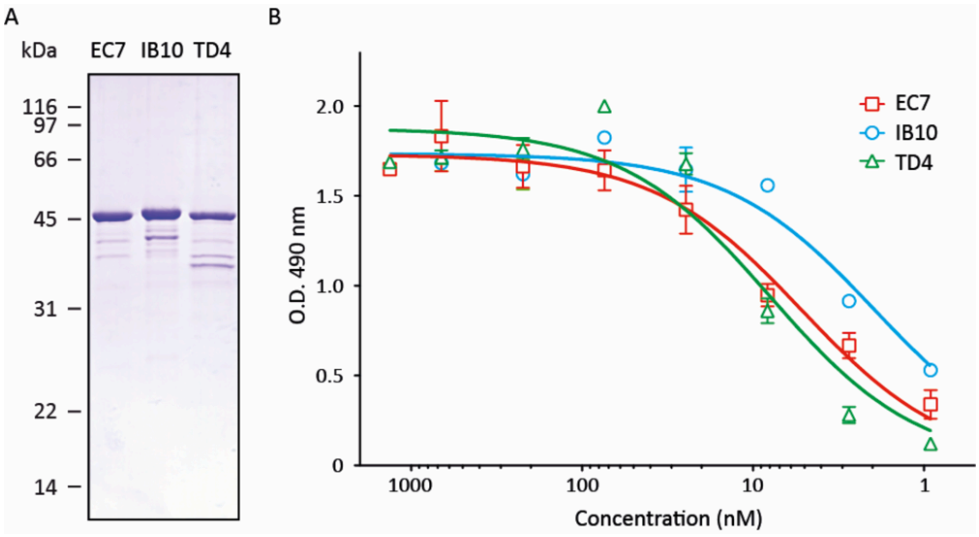


Figure 14. Purification and affinity determination of the selected V_{HH} clones from each library. A. Coomassie staining of the purified His-tagged V_{HH}-fusions. B. ELISA of 3-fold dilutions of the purifed V_{HH}-fusions, parting from 1 µM. Each concentration was tested doing triplicates.

Given that TD4 and IB10 recognise TirM and Int280, respectively, we evaluated whether they could interfere with their interaction. To elucidate this, we set up an *in vitro* assay of the Int280:TirM interaction, in which the binding of biotinylated Int280 (50 µg/ml) to TirM coating ELISA plates is revealed with Strepatavidin-POD (Figure 15). We found that addition of a 50 fold dilution of the immune camel serum and 1 µM (50 µg/ml) concentration of the purified V_{HH}-HlyA fusions TD4 and IB10 strongly competed with the Int280:TirM interaction. The signals of the Int280:TirM interaction were reduced ca. 5-fold in the case of IB10 and ca. 20-fold in the case of TD4. In contrast, no interference was observed when PBS, pre-immune serum and the purified V_{HH}-HlyA fusion EC7 (binding EspA) were added to the assay.

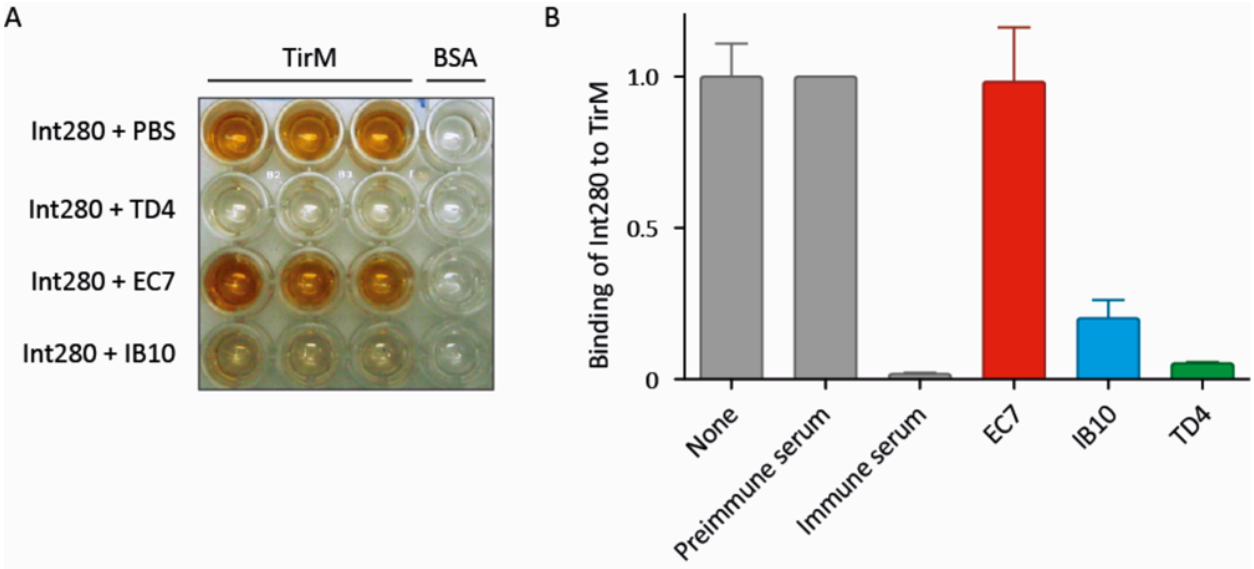


Figure 15. Competition assay of purified TD4, EC7 and IB10 with Int280 for the binding of TirM. A. ELISA showing the displacement of 50 µg/ml of biotynilated Int280 done by 50 µg/ml (1 µM) of each of the purified V_{HH}s. B. Representation of the obtained triplicate signals in relation to the addition of no V_{HH}. The camel serum was incubated using a 50 fold dilution.

Next, we tested whether the purified V_{HH}-HlyA fusions were capable of inhibiting the intimate attachment of EHEC to HeLa cells cultured *in vitro*. We hypothesized that TD4 and/or IB10 might block the formation of actin pedestals underneath the bound bacteria by inhibiting the Int:Tir interaction. On the other hand, EC7 might have an effect inhibiting the contact of EspA with host cells. To this end, HeLa cells were infected with EHEC at a MOI of 300:1 in the presence or not of a concentration of 1 µM of these V_{HH}-HlyA fusions. We selected this concentration because it was 10 times higher than the saturating concentration observed in ELISA. The formation of actin-rich pedestals by bacteria were evaluated by fluorescence confocal microscopy after staining with fluorophore-labelled phalloidin (actin), anti-O157 mAb (EHEC) and DAPI (DNA) (Figure 16). In the EHEC infections lacking purified V_{HH}-HlyA fusions, actin pedestals were evident underneath bacteria after 3 h of infection. An identical phenotype was observed when the V_{HH}-HlyA fusions EC7 and IB10 were added to EHEC infections. In contrast, EHEC infections incubated with TD4 showed no signs of actin accumulation beneath the attached bacteria, though EHEC was still bound to the HeLa cells. The bacteria were, however, loosely bound and a higher proportion of cells showed no surrounding

bacteria, possibly indicating that they were washed away. These data indicated that TD4 was also able to interfere with the Int:Tir interaction during infection of EHEC to HeLa cells, thereby blocking the intimate attachment of bacteria to the host cells.

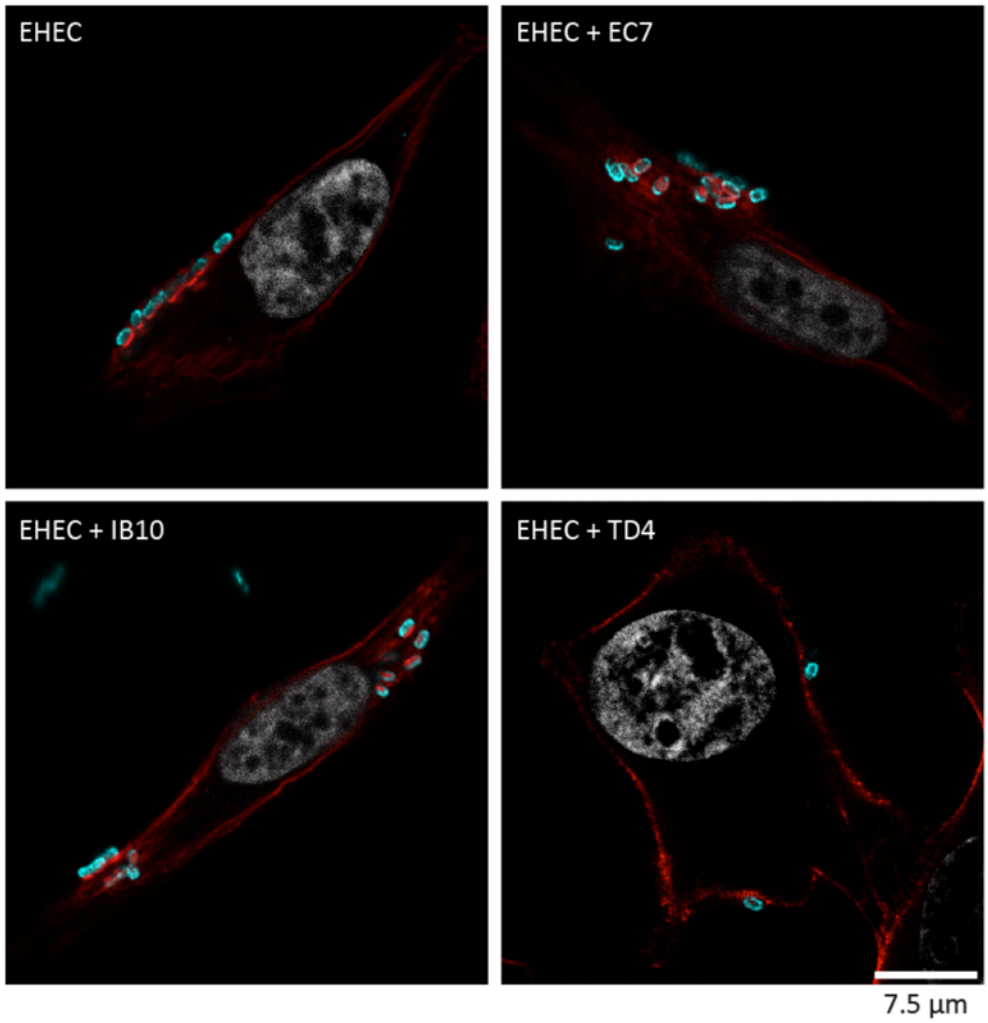


Figure 16. Infection of HeLa cells with EHEC with the addition of purified EC7, IB10 and TD4. Fluorescent staining of the actin cytoskeleton of the cells is shown in red, the DNA in gray and the O157 antigen of EHEC in cyan. High-magnification images of sample slices were taken at the confocal microscope after 3 h of infection. The upper-left panel shows the control infection of EHEC, with bound EHEC forming actin pedestals underneath the infection site. The rest of the panels shows the effect of the addition of EC7 (upper-right), IB10 (bottom-left) and TD4 (bottom-right) to the infection, all of them at a concentration of 1 µM.

2.2. Characterisation of the inhibition of EHEC intimate attachment to HeLa cells by TD4.

Given that TD4 was capable of inhibiting the intimate attachment of EHEC to HeLa cells *in vitro*, we further investigated its mechanism of action. First, we evaluated whether the clustering of Tir underneath bound EHEC bacteria was affected by the addition of TD4. HeLa cells were infected with EHEC, in the presence or not of TD4, and stained to reveal the presence of Tir with an anti-Tir_{EHEC} rabbit polyclonal serum, EHEC (O157), actin (phalloidin) and DNA (DAPI). Inspection of samples by fluorescence confocal microscopy (Figure 17) showed that addition of TD4 prevented the clustering of Tir as well as the formation of actin

pedestals underneath EHEC. As a control of the Tir staining, the anti-Tir_{EHEC} serum did not generate any significant fluorescence signal in HeLa cells infected with EHECΔ*tir*. Therefore, TD4 inhibits the clustering of Tir in the plasma membrane of the host cell by interfering with the Intimin-derived activation of Tir upon infection.

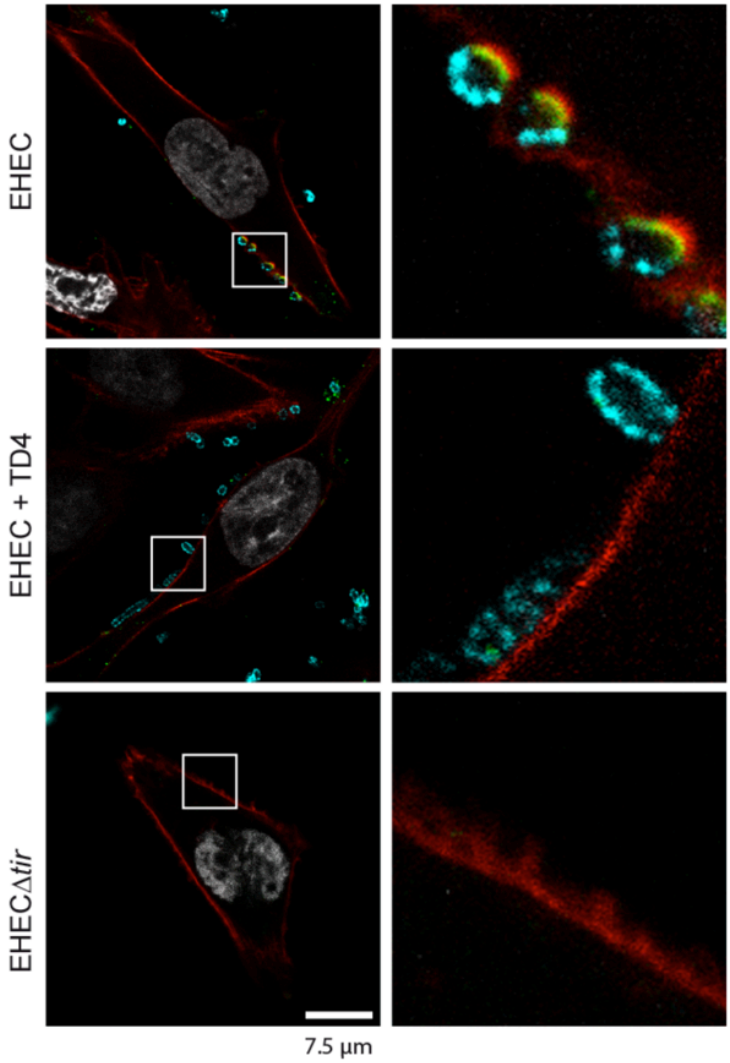


Figure 17. TD4-derived inhibition of the clustering of Tir upon EHEC infection. Fluorescent-staining of the actin cytoskeleton of the cells is shown in red, the DNA in gray, the O157 antigen of EHEC in cyan and Tir in green. High-magnification images of sample slices were taken at the confocal microscope after 3 h of infection. In the upper panels is represented the control infection of EHEC, the middle panels display the infection adding purified TD4 and the infection done by the mutant EHEC*Δtir* is depicted in the lower panels. The right panels are an amplification of the white square marked on the left panels.

Then, we assesed the inhibitory effect produced by TD4 in longer times of infection. To this end, HeLa cells were infected for 6 h with EHEC, in the presence or not of 1 µM TD4, and stained as above for their analysis by fluorescence confocal microscopy (Figure 18). Inspection of these samples revealed the presence of a very high number of EHEC bacteria intimately attached to the HeLa cells in the absence of TD4. These infected cells also showed the presence of dense clusters of Tir and actin associated with the attached bacteria. In contrast, the presence TD4 dramatically reduced the number of EHEC bacteria bound to HeLa cells, as well as the intensity of actin and Tir signals in the infected cells. HeLa cells infected with EHEC and

incubated with TD4 showed a pattern of actin filaments similar to HeLa cells infected with EHECΔ*tir* control, although weak accumulations of actin and Tir could be detected in some HeLa cells with bound wt EHEC bacteria.

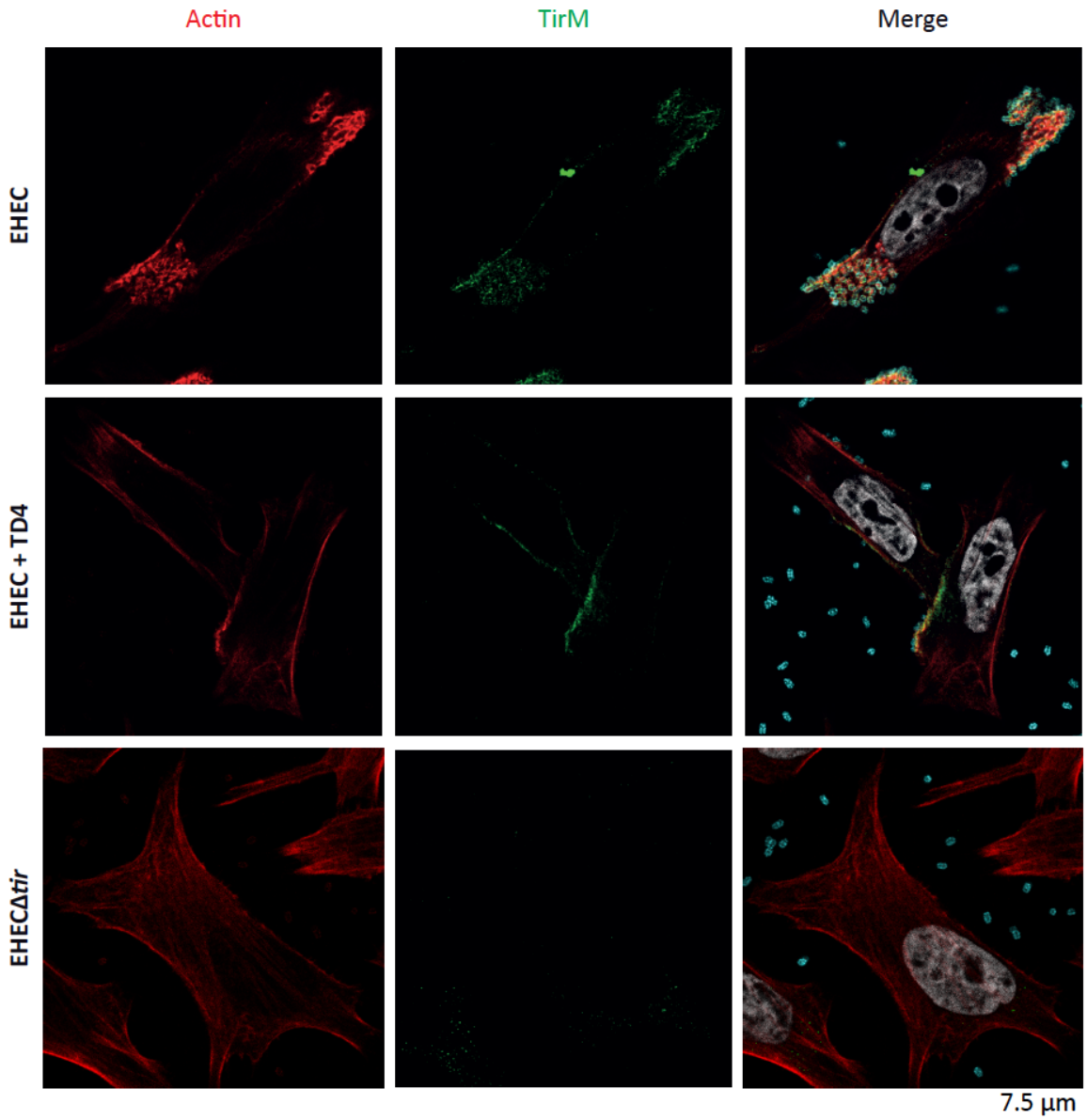


Figure 18. Effect of TD4 during a 6 h infection of EHEC. Fluorescent-staining of the actin cytoskeleton is shown in red, the DNA in gray, the O157 antigen of EHEC in cyan and Tir in green. High-magnification images of sample slices were taken at the confocal microscope after 6 h of infection. The upper panels show the infection control of EHEC wt; the middle panels, the effect of TD4 to this infection; and the panel in the bottom, the infection of the EHEC*Δtir* mutant.

2.3. Comparison of the binding affinities of TD4 and Int280 for TirM

The results described above indicated that the V_{HH}-HlyA fusion TD4 competes with Int for the binding of Tir on the cell surface, blocking Tir clustering on the host plasma membrane and abolishing the downstream Tir-dependent signalling that leads to the actin polymerization. Next, we compared the affinities of Int280 and TD4 for TirM using surface plasmon resonance (SPR). Biotinylated TirM was immobilised onto a

streptavidin-coated gold chip for SPR, and purified Int280 and TD4 were passed through this chip at different concentrations in succesive rounds of binding and regeneration (Figure 19A). The change in resonance units (RUs) with time was recorded as a direct indication of the binding of these proteins to TirM and was used to generate the sensograms represented in Figures 19 and 20.

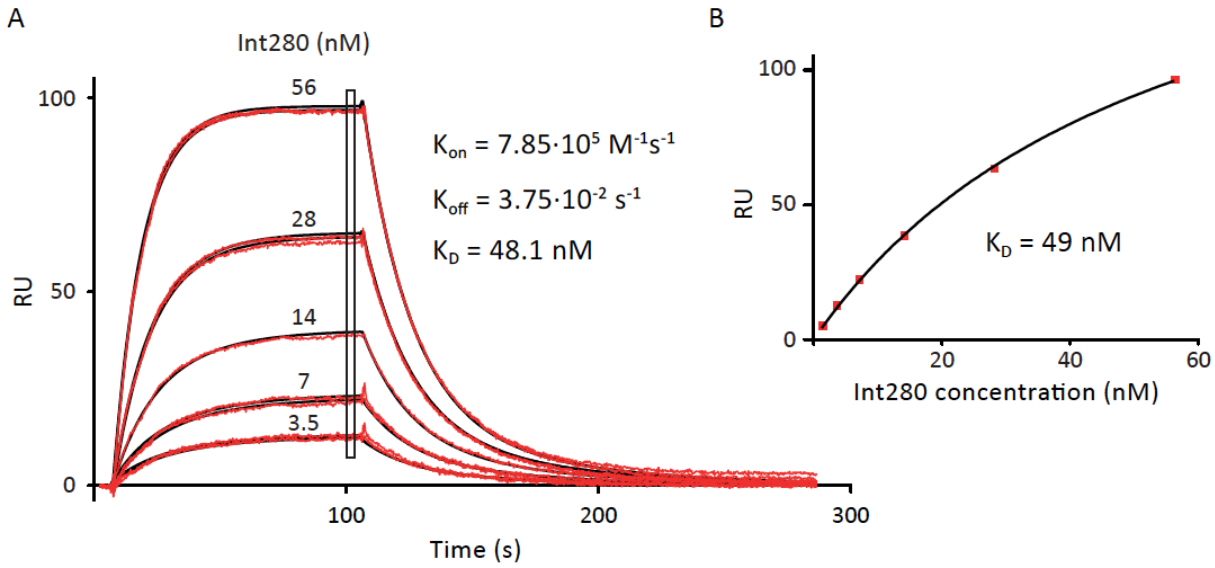


Figure 19. SPR analysis of Int280. A. Indicated concentrations of purified Int280 passed through a streptavidin chip coated with biotinylated TirM to calculate the K_{ON}, K_{OFF} and K_D. B. For a second estimation of the K_D, the RUs recorded at the steady state were analyzed using the BiaEvaluation software.

These experiments revealed a distinct pattern of TirM binding by Int280 and TD4. While Int280 quickly bound to and dissociated from TirM, TD4 bound to TirM more slowly and the interaction remained stable without any detectable dissociation, even >300 sec after stopping the injection of the protein. No binding was observed when an unrelated control V_{HH} (binding GFP) was passed through the sensor (data not shown). The kinetic constants of Int280 binding TirM could be calculated directly from the obtained sensograms (Figure 19A). A model 1:1 Langmuir interaction perfectly fitted the binding curves, suggesting the formation of a 1:1 complex, as observed by protein crystallography with Int280-TirM from EPEC[214,399]. Using this binding model, we determined a k_{off} of 3.75·10^{-2} s^{-1} and a k_{on} of 7.85·10^5 M^{-1}s^{-1}. The equilibrium dissociation constant (K_D) was calculated from the kinetic constants (k_{off}/k_{on}) and determined to be 48.1 nM. The K_D of the Int280:TirM interaction was also calculated by plotting the RUs at the steady state observed with the different concentrations of Int280, which fitted with an apparent K_D of 49 nM (Figure 19B). In contrast to Int280, the fact that TD4 had no detectable dissociation of TirM during SPR analysis impeded the determination of its kinetic constants k_{on} and k_{off} from the obtained sensograms. In addition, the K_D could not be precisely determined from RU values of TD4 binding since the steady state was only reached at the highest concentration of TD4. Using the RU values at the apparent steady state of the different concentration tested, we could calculate an apparent K_D of 4.8 nM for the TD4:TirM interaction (Figure 20B). The actual K_D for this interaction it likely to be below this estimated value (K_D < 4.8 nM) as the

steady state would be reached with higher RU values. Hence, this quantitative binding analysis indicated an at least 10-fold higher affinity of the TD4-HlyA fusion for TirM than the one of its natural ligand, Int280.

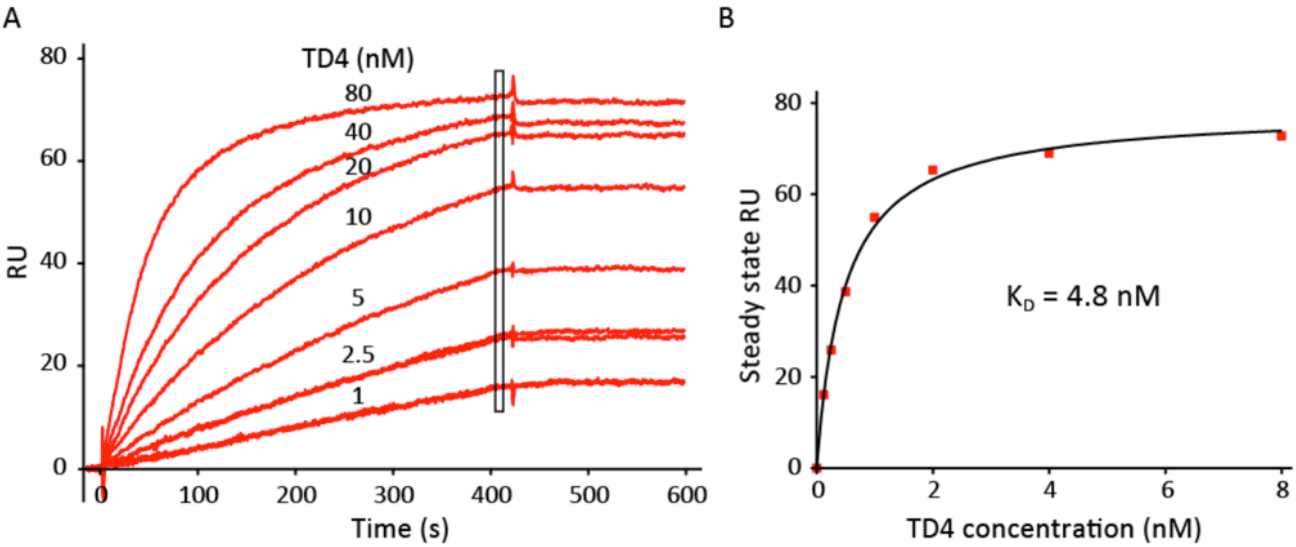


Figure 20. SPR analysis of TD4-Hly. A. Indicated concentrations of purified TD4-Hly fusions passed through a streptavidin chip coated with biotinylated TirM. The association constant (K_{ON}) could not be calculated due to the very low dissociation constant (K_{OFF}). B. The obtained RU shift at the steady state was used to calculate the apparent affinity constant (K_D) using the BiaEvaluation software.

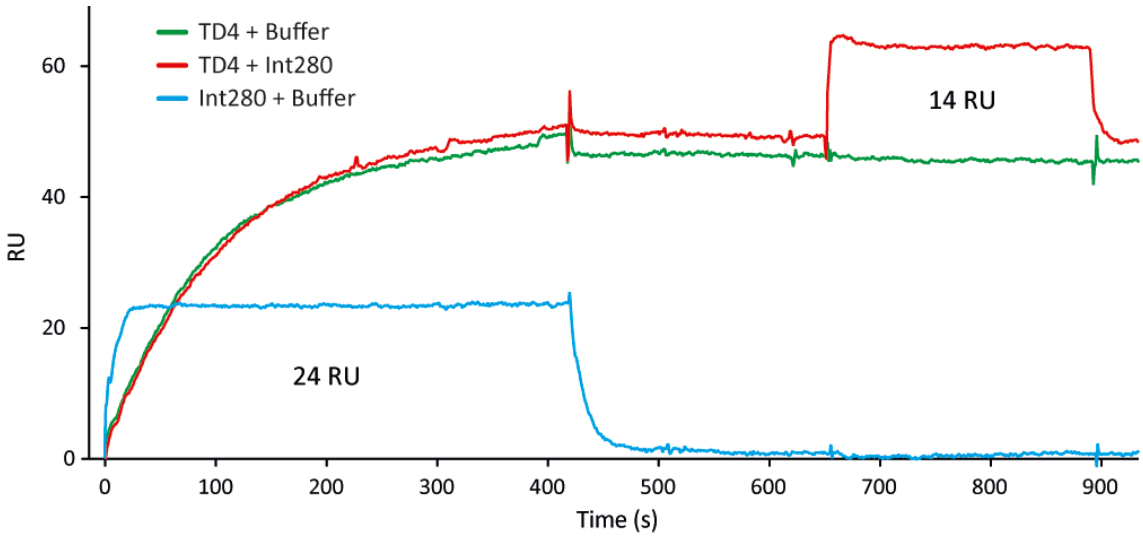


Figure 21. Competition of Int280 and TD4-Hly in a surface plasmon resonance analysis. A concetnration of 40 nM of TD4 followed by Hepes buffer (green), TD4 followed by 80 nM Int280 (red) or Int280 followed by Hepes buffer (blue) were subjected tor TirM binding. The RU shift showed by the binding of Int280 to TirM was compared with the RU shift obtained once TD4 was previously bound.

Using SPR we investigated whether TD4 recognises an epitope of TirM that overlaps the binding site of Int280, taking advantage of the extremely slow dissociation of TD4. We injected 40 nM of TD4-HlyA into the TirM-chip until reaching RU values close to steady state followed by 80 nM of Int280 (Figure 21). We compared the increment of RU values obtained by Int280 injection in this condition (with bound TD4) with those obtained by injecting the same concentration of Int280 to the TirM-chip in the absence of TD4. This experiment showed that the RU values of Int280 binding to TirM were reduced by 2-fold in the presence of

TD4, but binding of Int280 occurred simultaneously to TD4, indicating that the binding sites of Int280 and TD4 cannot be identical, although they may partially overlap. Interestingly, the k_{on} of Int280 seems to be higher than the one of TD4, since it reached the steady state more quickly than the V_{HH}-HlyA fusion.

Next, we wanted to find out the specific binding site of TD4 to TirM. We synthesized 12-mer peptides of TirM covering its sequence, with a 10 amino acid overlap between consecutive peptides on a PVDF membrane for the incubation with TD4-HlyA. Bound TD4-HlyA was detected with anti-E-tag mAb identifying two peptides recognised by TD4: VNIDELGNAIPS (amino acids 296 - 307) and GVLKDDVVANIE (amino acids 308 - 319) (Figure 22). These peptides are consecutive in TirM sequence, and localized within the interaction interface between EPEC TirM and Int found by crystallography[214] (Figure 23).

EHEC TirM 12-mer peptides

1	QALALTPEPDSP	11	ASATETATRDQL	21	DNQKVNIDELGN	31	DDVVANIEEQAK	41	QAIENNAQAQKK
2	LALTPEPDSPTT	12	ATETATRDQLTK	22	QKVNIDELGNAI	32	VVANIEEQAKAA	42	IENNAQAQKKYD
3	LTPEPDSPTTTD	13	ETATRDQLTKEA	23	VNIDELGNAIPS	33	ANIEEQAKAAGE	43	NNAQAQKKYDEQ
4	PEPDSPTTTDPD	14	ATRDQLTKEAFQ	24	IDELGNAIPSGV	34	IEEQAKAAGEEA	44	AQAQKKYDEQQA
5	PDSPTTTDPDAA	15	RDQLTKEAFQNP	25	ELGNAIPSGVLK	35	EQAKAAGEEAKQ	45	AQKKYDEQQAKR
6	SPTTTDPDAAAS	16	QLTKEAFQNPDN	26	GNAIPSGVLKDD	36	AKAAGEEAKQQA	46	KKYDEQQAKRQE
7	TTTDPDAAASAT	17	TKEAFQNPDNQK	27	AIPSGVLKDDVV	37	AAGEEAKQQAIE	47	YDEQQAKRQEEL
8	TDPDAAASATET	18	EAFQNPDNQKVN	28	PSGVLKDDVVAN	38	GEEAKQQAIENN	48	EQQAKRQEELKV
9	PDAAASATETAT	19	FQNPDNQKVNID	29	GVLKDDVVANIE	39	EAKQQAIENNAQ	49	QAKRQEELKVSS
10	AAASATETATRD	20	NPDNQKVNIDEL	30	LKDDVVANIEEQ	40	KQQAIENNAQAQ		

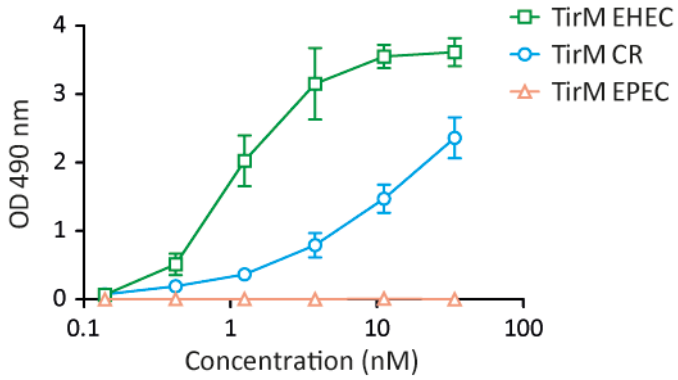
Figure 22. Identification of the TirM sequence recognised by TD4-Hly. A set of 12-mer peptides overlapping 10 residues and reconstructing the sequence of TirM were synthesized on a PVDF membrane and were incubated with purified TD4-HlyA. Those peptides to which TD4-HlyA was bound were detected with anti-E-tag mAb.

TirM is a protein fragment that is highly conserved among the related pathogens EHEC, EPEC and *C. rodentium* (CR). We purified His-tagged versions of TirM from EPEC and CR to test the affinity of TD4 against them in ELISA in comparison to TirM of EHEC (Figure 24A). We found that TD4 bound CR TirM with lower affinity (ca. 10-fold) than EHEC TirM. Surprisingly, TD4 did not bind EPEC TirM at all, indicating that the minor differences in their sequence compared to EHEC TirM abrogate the interaction with TD4. We compared the sequences of the TD4 binding sites of EHEC TirM with the corresponding ones of EPEC and CR (Figure 24B). Within the recognized peptides, EHEC TirM diverges with both EPEC and CR sequences in amino acids V309E, N317Q and E319A, which suggests that changes in these amino acids reduce the affinity of TD4 towards TirM. Moreover, EHEC TirM specifically differs with EPEC TirM (but not with CR TirM) in amino acids E300A, L301N, V314I and A316E, suggesting that these residues may be essential for TirM recognition by TD4.

TirM-EHEC AASATETATRDQLTKEAFQNPDNQKVNIDELGNAIPSGVLKDDVVANIEEQAKAAGEEAKQQA

Figure 23. Recognition interface of TirM by Int280. Structure of the crystal 1F02 (PDB) of the interaction site of Int (in pink) and Tir (in white) of EPEC. The peptides of EHEC TirM identified to be recognised by TD4 are coloured in green and blue both in the crystal representation and in the linear sequence of the TirM domain of EHEC.

A



B

TirM-EPEC AANAAESATKDQLTQEAFKNPENQKVNIDANGNAIPSGELKDDIVEQIAQQAKEAGEVARQQA
TirM-CR AANAAEAATKDQLTKEAFQNPDNQKVNIDELGNAIPSGELKDDVVAQIADQAKVAGEQARQQA
TirM-EHEC AASATETATRDQLTKEAFQNPDNQKVNIDELGNAIPSGVLKDDVVANIEEQAKAAGEEAKQQA

Figure 24. Recognisition of the TirM homologs in EHEC, EPEC and CR by TD4-HlyA. A. Indicated concentrations of purified TD4-HlyA were tested in ELISA against these proteins. B. Alignment of the sequences of the TirM domains of EHEC, EPEC and CR. In green and blue are coloured the peptides recognised by TD4-HlyA in EHEC. Inside the red rectangle are depicted the identified aminoacids for the Int:Tir interaction in EPEC[299]. Highlighted in red are the aminoacids to be essential for the binding of TD4-HlyA and in yellow, those that reduce the affinity.

In summary, these results demonstrated that TD4-HlyA binds to EHEC TirM with high affinity (K_D < 4.8 nM), directly recognizes the peptide sequence VNIDELGNAIPSGVLKDDVVANIE (296-319) and overlaps with the binding site of Int280, which has a lower affinity (K_D 48 nM) and a faster dissociation rate from TirM. TD4

binds TirM of CR with significant lower affinity, whereas TirM of EPEC is not recognized due to specific amino acid changes compared to EHEC TirM.

3. Engineering of *C. rodentium* to express operon LEE5 of EHEC.

EHEC infects humans, but has its reservoir in cattle and other ruminants, which they colonise in the terminal rectum[258]. Despite its importance, there is still no convenient animal model for the study of the A/E lesion formation during the infection of EHEC. Large ruminants are costly to maintain and require specialized facilities for experimentation, whereas adult mice and rabbits do not develop A/E lesions after EHEC infection. Only newborn infant rabbits not colonized by other bacteria have been shown to develop A/E lesions after EHEC infection[296]. However, this model is difficult to establish given the strict aseptic conditions that need to be employed and the limited time window of infection, whithin the first days of life of the animals. In addition, this model cannot reflect the infection of adults and the colonization of mucosa with natural microbiota. The lack of a naturally ocurring strain of EHEC able to colonise the healthy intestine of mouse models - more prone for the use in the laboratory - hinders the testing of therapies that would limit the infection outbreaks caused by EHEC. For this reason, the mouse-restricted pathogen CR serves as a surrogate animal model for the infection of EHEC.

We did not observe inhibition of actin pedestals formed by CR on HeLa cells upon addition of TD4 (data not shown), likely due to the low affinity of this sdAb for TirM of CR. Therefore, in order to test TD4 inhibition of A/E lesions in an *in vivo* mouse model, we generated a recombinant strain of CR expressing Int and Tir of EHEC. For this we used a CR strain expressing the EHEC effector TccP) from the chromosome (CR-Tccp)[119], which is required for coupling Tir to actin pedestal formation in EHEC[114]. Before introducing the LEE5 operon of EHEC, which harbours Int, Tir and the chaperone CesT, we deleted the homologous LEE5 operon from CR-TccP strain, generating CR-TccPΔLEE5. This deletion and the subsequent integration of EHEC LEE5 was done by means of a double recombination strategy using thermo-sensitive suicide plasmids pGETS (see methods). The resulting CR strain was named CR-EHEC, which harbours the EHEC LEE5 operon and TccP inserted in the chromosome of CR.

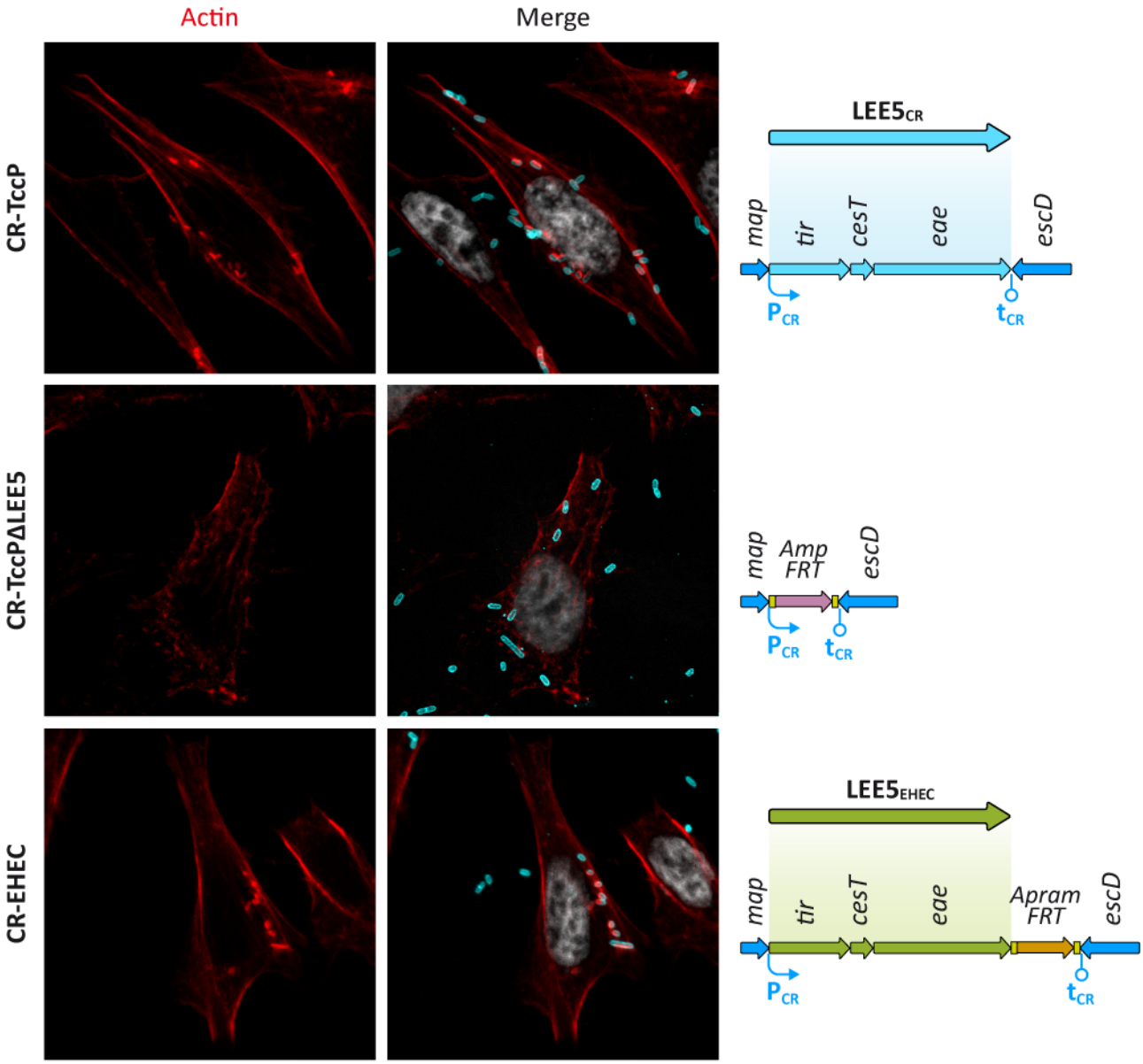


Figure 25. Infection of CR-TccP, CR-TccPΔLEE5 and CR-EHEC on HeLa cells. Fluorescent-staining of the actin cytoskeleton is shown in red, the DNA in gray and CR in cyan. High-magnification images of the samples were taken at the confocal microscope after 3 h of infection. CR-TccP (upper panels), CR-TccPΔLEE5 (middle panels) and CR-EHEC (bottom panels) tested for the formation of actin pedestals.

We assessed the ability of CR-EHEC to form actin pedestals on HeLa cells by confocal microscopy using CR-TccP and CR-TccPΔLEE5 as positive and negative controls, respectively (Figure 25). All of the strains adhered to the HeLa cells but, as expected, CR-TccPΔLEE5 did not form actin pedestals on the cell surface, whereas CR-TccP and CR-EHEC were able to form actin pedestals of similar morphology. These results demonstrated that Int and Tir of EHEC were functional in CR-TccP to assemble actin pedestals. We then infected HeLa cells with CR-TccPΔLEE5 (negative control) and CR-EHEC and tested the addition of TD4 or a control V_{HH}-HlyA fusion (Vamy) to CR-EHEC (Figure 26). In the presence of TD4, CR-EHEC adhered to HeLa cells but did not form actin pedestals beneath the bacteria.

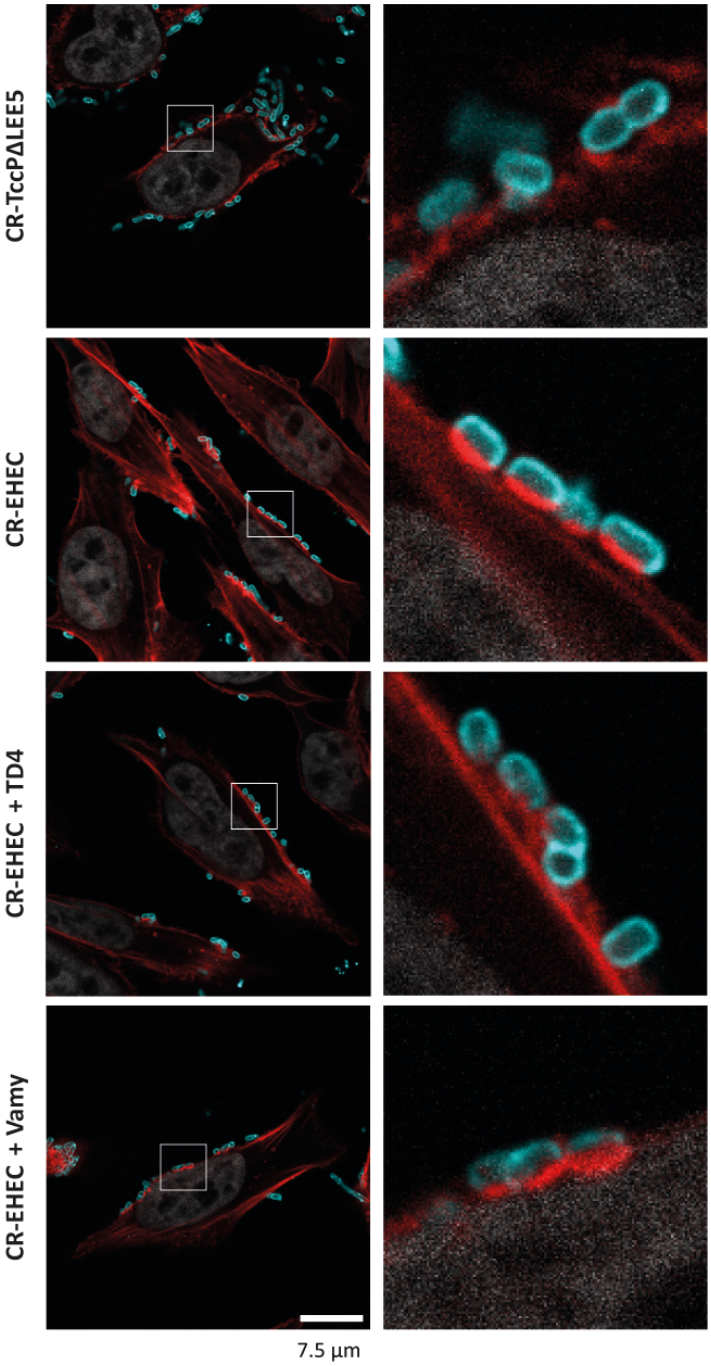


Figure 26. Addition of TD4 to the infection of CR-EHEC on HeLa cells. Fluorescent-staining of the actin cytoskeleton is shown in red, the DNA in gray and CR in cyan. High-magnification images of sample slices were taken at the confocal microscope after 3 h of infection. TD4 (middle-down pannel) and Vamy (bottom panel) were added with CR-EHEC to HeLa cells to measure the inhibiting activity of TD4. CR-TccPΔLEE5 (upper pannel) and CR-EHEC alone (middle-up pannel) were tested as controls.

In collaboration with the laboratory of Prof. Gad Frankel (Imperial College London, UK) we tested whether CR-EHEC was able to colonise the mice intestine and form actin pedestals *in vivo*. Pathogen-free female C57BL/6 mice (Charles River; n=6 per group) were infected with $1x10^9$ CFU of CR or CR-EHEC and the number of bacteria per gram of stool were followed at days 2, 4, 7 and 8 post-inoculation (Figure 27). Infected mice showed colonisation of the gastrointestinal tract with both strains, as $>10^8$ CFU were detected in the stools of infected mice of both groups after 1 week post-inoculation, indicating that CR-

EHEC was able to colonize and infect the mouse intestine and produce diarrhoea. Though a lower number of CR-EHEC than CR were found in stools at short times post-inoculation (i.e. 2 days), this decrease is usual shortly after the inoculation, since the inoculated non-bound bacteria are washed from the intestine. Finally, histological cross-sections of colonic tissue from mice of each group were analyzed 8 days post-infection to determine the generation of hyperplasia in the intestinal crypts (Figure 27).

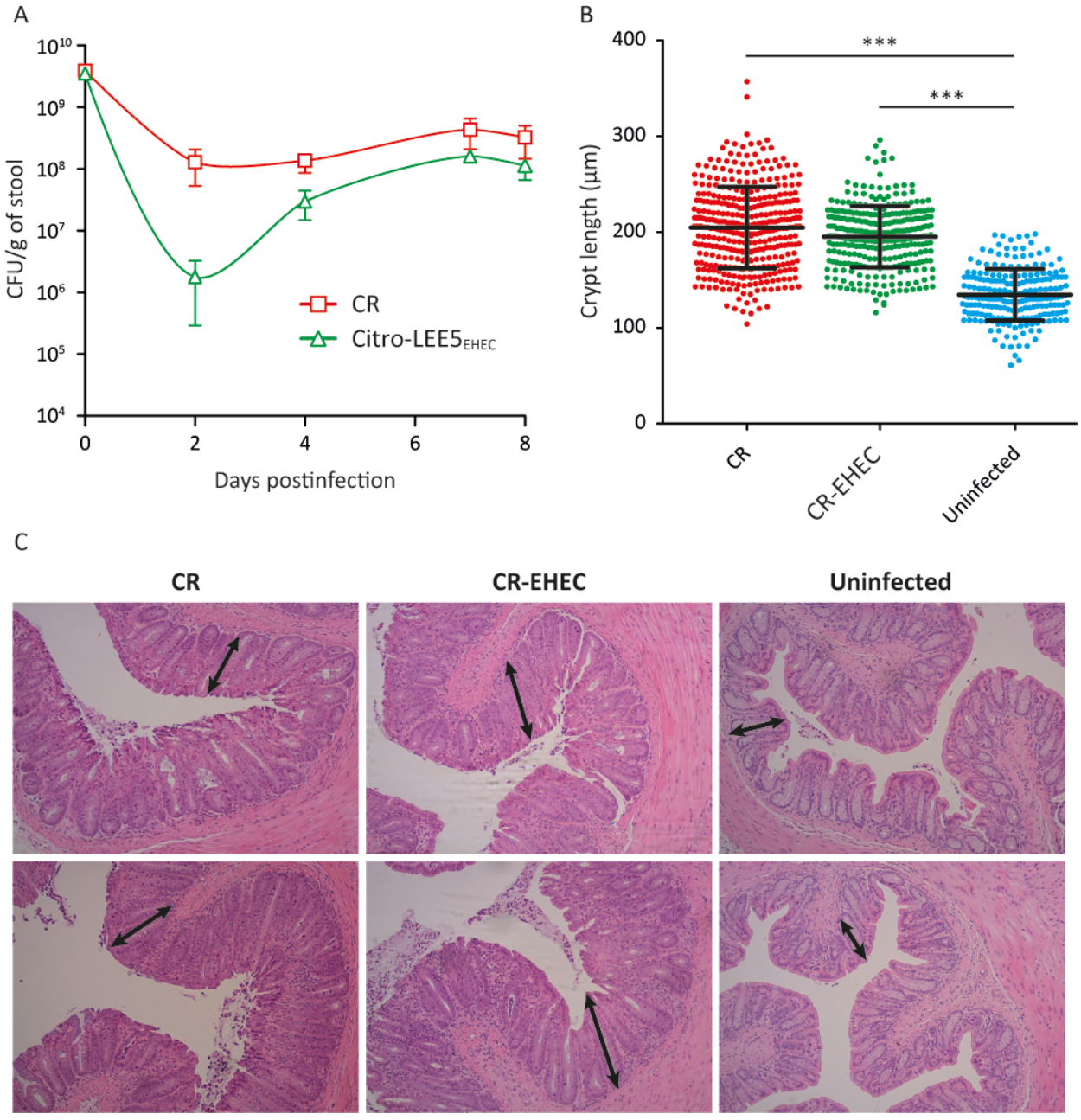


Figure 27. Infection of CR-EHEC in the mouse intestine. A. Two groups of 6 mice were infected with a dose of $6{\cdot}10^9$ bacteria. The CFUs per gram of stool were counted up to 8 days postinfection. B and C. Two groups of 6 mice were infected with CR and CR-EHEC and the length of the intestinal crypts in the colonic tissue was measured 8 day postinfection in comparison with a group of 4 uninfected mice.

This analysis revealed that the intestinal crypts from animals infected with CR and CR-EHEC had a similar length (205 µm), significantly longer than the mean lenght of crypts form uninfected control animals (135

μm). These results demonstrate that CR-EHEC strain is able to colonize and infect the intestine of mice inducing diarrhoea and colonic hyperplasia similar to wild type CR. Hence, the engineered CR-EHEC strain could be used *in vivo* using infected adult mice to test the effect of TD4 in the formation of A/E lesions mediated by Int and Tir proteins of EHEC. These experiments are currently being planned and will be conducted in collaboration with the laboratory of Prof. Gad Frankel.

4. Engineering *E. coli* K-12 for the expression of the HlyA secretion system from the chromosome

The efficacy of the effect of TD4 to prevent the formation of actin pedestals upon the infection of EHEC and the generation of CR-EHEC, that can be used for the study of the effect of TD4 *in vivo*, led us to engineer an *E. coli* K-12 commensal strain to express the HlyA secretion system from the chromosome. To this end, we used the same strategy as for the modification of CR. First, we cloned the TD4-HlyA fusion protein into the pVDL9.3 plasmid, thereby generating a sole operon regulated under the Plac promoter that harbours the V_{HH}-HlyA fusion, HlyB and HlyD. We demonstrated that this plasmid can secrete TD4 to the medium and that it can recognise TirM in an ELISA (data not shown). This cassette was then cloned devoid of promoter into a pGETS plasmid harbouring homology regions for the integration into the *yeeJ* chromosomal site (see Chapter 2 for the characterization of this integration site). For a controlled expression of the fusion *in vivo*[209], we cloned the PBAD promoter[130] - inducible with arabinose - upstream of the operon. The integration of the TD4-HlyA operon was done by means of a double recombination strategy, as above, in a generated MG1655 strain lacking the AraC gene (see methods). The resulting strain was named HS-TD4. As a negative control, we integrated the construct lacking the PBAD promoter in the same strain (HSΔp-TD4).

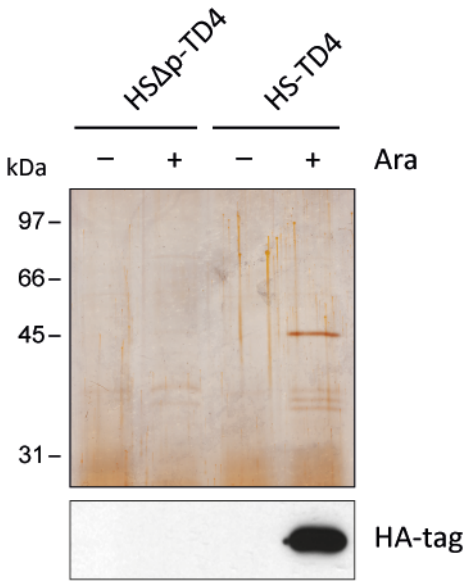


Figure 28. Secretion of TD4-HlyA by strain HS-TD4. Cultures of HSΔp-TD4 and HS-TD4 strains were grown with or without the presence of arabinose to test the secretion of TD4-HlyA to the medium. The culture supernatants were analyzed by silver staining and Western blot using an anti-HA-tag mAb.

Next, we cultured HS-TD4 and HSΔp-TD4 in liquid LB and with or without the presence of arabinose. We analyzed the culture supernatant by silver staining and Western blot (Figure 28) and determined that the TD4-HlyA fusion was only secreted by HS-TD4 in the presence of arabinose, while we could not detect secretion done by HSΔp-TD4. We also measured the growth of this strain to determine if the expression of the HlyA operon could be hindering the growth of HS-TD4 (Figure 28). We observed that the induction with arabinose retarded the growth of HS-TD4 mildly.

Chapter 2: Controlled assembly of the T3SS of EPEC in the non-pathogenic *E. coli* K-12

T3SSs are exclusively present in pathogenic bacterial strains but could be used as a tool for the delivery of therapeutic proteins to the cytoplasm of human and other mammalian cells if assembled in non-pathogenic bacteria, such as commensal and probiotic strains, and this is the major objective pursued in this chapter of the PhD thesis.

1. Injection of V_{HH}s to the cytoplasm of mammalian cells with EPEC

In our laboratory, it was previously demonstrated that the T3SS of EPEC was capable of secreting functional V_{HH}s to the culture medium and injecting them directly into the cytoplasm of HeLa cells[29]. In these experiments two V_{HH}s binding model antigens (GFP and α-amylase) were fused to a T3-secretion signal corresponding tothe N-terminal 20 amino acids of EPEC effector EspF. Although secretion and translocation of both VHH clones was sucessful in wild-type and attenuated EPEC strains carrying a functional T3SS, we wanted to further test the versatility of this T3SS for the translocation of additional V_{HH}s before planning its transplantation to a non-pathogenic *E. coli* strain, such as K-12 MG1655[31].

We received two V_{HH}s binding the human cytoplasmic protein CapG from the laboratory of Prof. Jan Gettemans (University of Ghent, Belgium). CapG is a member of the actin regulatory proteins able to bind and block the barbed ends of F-actin, contributing to the control of actin-based motility[367,389]. We cloned these V_{HH} sequences, named VcapG2 and VcapG4, in the vector pT3sVgfp[29] replacing Vgfp sequence. The resulting plasmids, pT3s-VcapG2 and pT3s-VcapG4, encode fusion proteins of these VHHs to the N-terminal T3-secretion signal of EspF and C-terminal His- and E-tag epitopes used for immunodetection. Expression of the T3s-VHH fusions can be induced in these vectors from an upstream Ptac promoter by addition of IPTG.

A functional T3SS in EPEC bacteria can be easily monitored by the presence of the translocators EspA, EspB and EspD[29] in culture supernantants of bacteria grown at 37°C in DMEM Plasmids encoding T3sVgfp, T3sVcapG2 and T3sVcapG4 were transformed into the attenuated EPEC strain Quad (EPECquad), which is a quadruple deletion mutant in Int and the LEE effectors Tir, EspF and Map (REF), and the EPEC mutant strain EPEC*ΔescN*, which lacks the specific ATPase EscN of the T3SS. The resulting transformed bacteria were grown in DMEM at 37°C to stimulate the expression of the T3SS and induced with 0.1 mM IPTG for 3 hours to allow the expression of the T3s-V_{HH}fusions. To analyze the presence of the V_{HH}s, we precipitated the proteins of the supernatants to concentrate them (Figure 29). Using SDS-PAGE and Coomassie blue staining of the proteins, we detected bands in EPECquad corresponding in size with the protease EspC (100 KDa) and the translocators EspA (26 KDa), EspB (35 KDa) and EspD (39 KDa). In the case of the T3S defective

strain EPEC*ΔescN*, we detected only detected the protease EspC, that is T3SS-independent. We detected the V_{HH}s and EspA by Western blot in the supernatants of EPEC quad but not EPEC*ΔescN*, indicating that they had been secreted by the T3SS. The bacterial lysates showed the expression of the E-tagged V_{HH}s in all of the cultures.

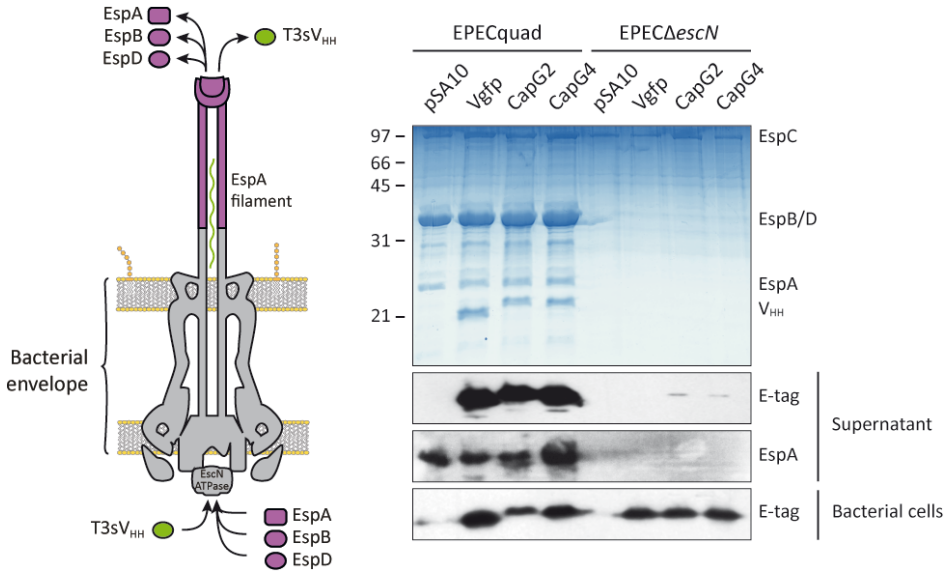


Figure 29. Secretion of anti-CapG V_{HH}s using the T3SS. Coomaise staining and Western blots of the supernatants of EPECquad and EPEC*ΔescN* harboring plasmids for the secretion of four different anti-CapG V_{HH}s and Vgfp. The secretion by the T3SS was confirmed by Western blot with anti-Etag and anti-EspA antibodies of the supernatants. The expression of the V_{HH}s was checked in the bacterial extract with an anti-Etag antibody.

Next, we tested if these V_{HH}s could be injected into the cytoplasm of the mice breast cancer cell line MDA-MB-231. To this end, we employed an assay that makes use of the enzymatic activity of the b-lactamase (Bla) to indicate and measure the translocation (Figure 30A). The substrate CCF2/AM is transformed to CCF2 inside the cells emiting green flourescence at 520 nm. When the b-lactamase is translocated inside the cell it is hydrolyzed and shifts its fluorescence to blue (450 nm). We cloned the T3s-V_{HH} fusions in plasmid pCX340, controlled under the Ptac promoter and fused to the b-lactamase at the C-terminal, and transformed EPECquad and EPEC*ΔescN* with the resulting plasmids pT3s-VcapG2-Bla and pT3s-VcapG4-Bla. We also transformed EPEC with the positive control plasmid pT3SVgfp-Bla and the empty vector pCX340, which harbours no T3S signal and is unable to translocate the b-lactamase, and induced DMEM cultures with IPTG. We infected the cells during 90 min and then incubated them with the CCF2/AM substrate to detect the translocation of the fusions. We observed at the fluorescent microscope that all of the EPEC infected cells emitted blue flourescence, indicating translocation; while the EPEC*ΔescN* infected cells emitted green fluorescence (Figure 30B). We also measured the catalitic activity of the b-lactamase in three independent experiments calculating the blue to green fluorescence emission ratio using a Fluostar Optima fluorescence reader (Figure 30C). VcapG2 and VcapG4 showed higher emission ratio than the Vgfp controlTherefore, attenuated EPEC bacteria with a functional T3SS can inject VcapG antibodies into the cytoplasm of MDA-MB-231 cells.

Then, we transformed EPEC with pT3SVcapG4 and pT3SVgfp as a control to investigated whether the injection of any of this anti-CapG antibody could cause some phenotypic effect in the formation of pedestals, given that CapG binds F-actin[364] and EPEC rearrange the actin cytoskeleton during its infection[177]. We infected HeLa cells with the induced strains, stained the actin cytoskeleton and observed the results at the flourescence microscope. In comparison with EPEC harbouring Vgfp, which showed no difference in the phenotype of the pedestals of wild type EPEC, the injection of VcapG4 showed a clear alteration of the actin pedestals. They were highly elongated structures separated from the main body of the cell through long projections of the host cell plasma membrane. Wild type EPEC bacteria, or those bacteria injecting Vgfp showed a wild type infection phenotype, with F-actin accumulation restricted to the intimate attachment sites and absence of membrane projections (Figure 31). This indicates that EPEC is capable of secreting and injecting a wide range of V_{HH} sequences. VcapG4 is even capable of interfere with the actin-cytoskeleton.

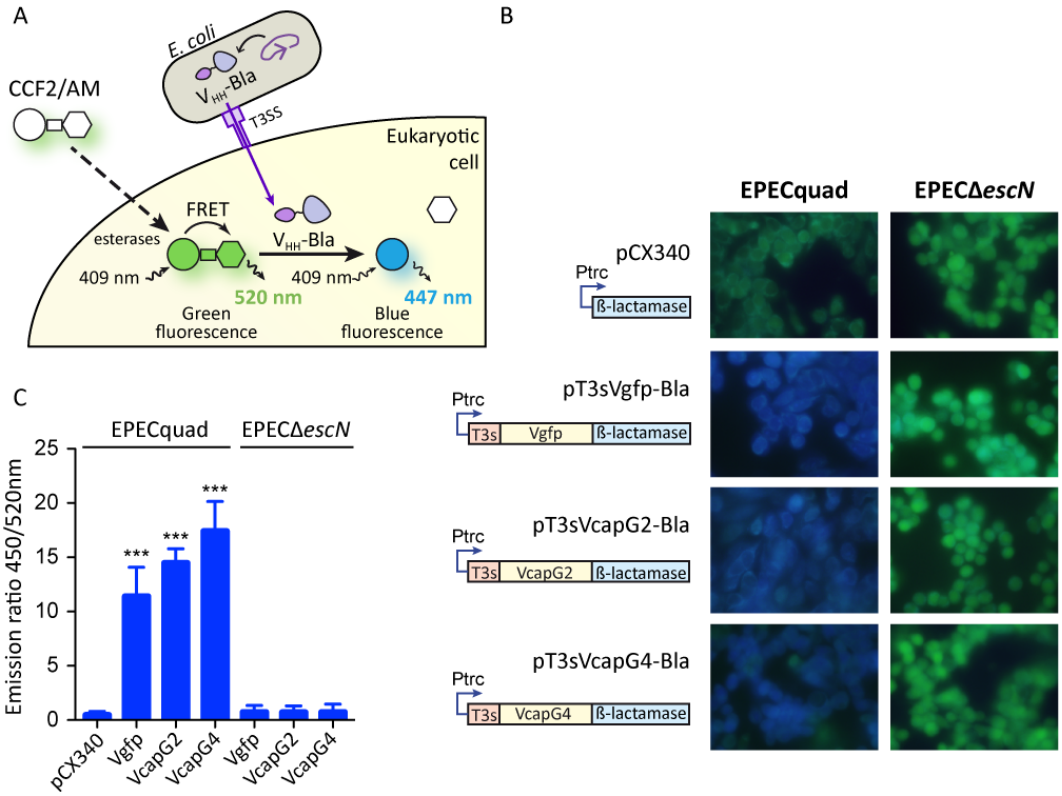


Figure 30. Translocation assay of VcapG-Bla fusions to the cytoplasm of MDA-MB-231 cells. A. Scheme describing the injection of a V_{HH} fused to a T3-secretion signal (T3s) and β-lactamase (Bla) reporter into a mammalian cell by the T3SS of enteropathogenic *E. coli* (EPEC) strains. The non-fluorescent Bla substrate (CCF2/AM) is modified by cytoplasmic esterases to the green fluorescent substrate CCF2. CCF2 hydrolysis by Bla results in a shift of green to blue fluorescence. B. Fluorescence observed in the infection with attenuated EPEC bacteria (quad) carrying T3s-Bla fusions of GFPNb (positive control), VcapG2 and VcapG4. Green fluorescence is observed in MDA-MB-231 cells infected with *ΔescN* strains (lacking the essential ATPase of the T3SS) or a Bla construct lacking the T3s signal sequence (pCX340). C. Emission ratio of the blue (450nm) and green (520 nm) fluorescence signals obtained from the fluorimeter. Results are expressed as mean ± SEM of three independent infection experiments for every bacterial strain. Data were analyzed by using one-way analysis of variance (ANOVA) and Bonferroni Multiple Comparison Test with the PRISM software (Graphpad) to calculate the P values of each data set with the control (quad pCX340). Asterisks (***) indicate $P < 0.001$.

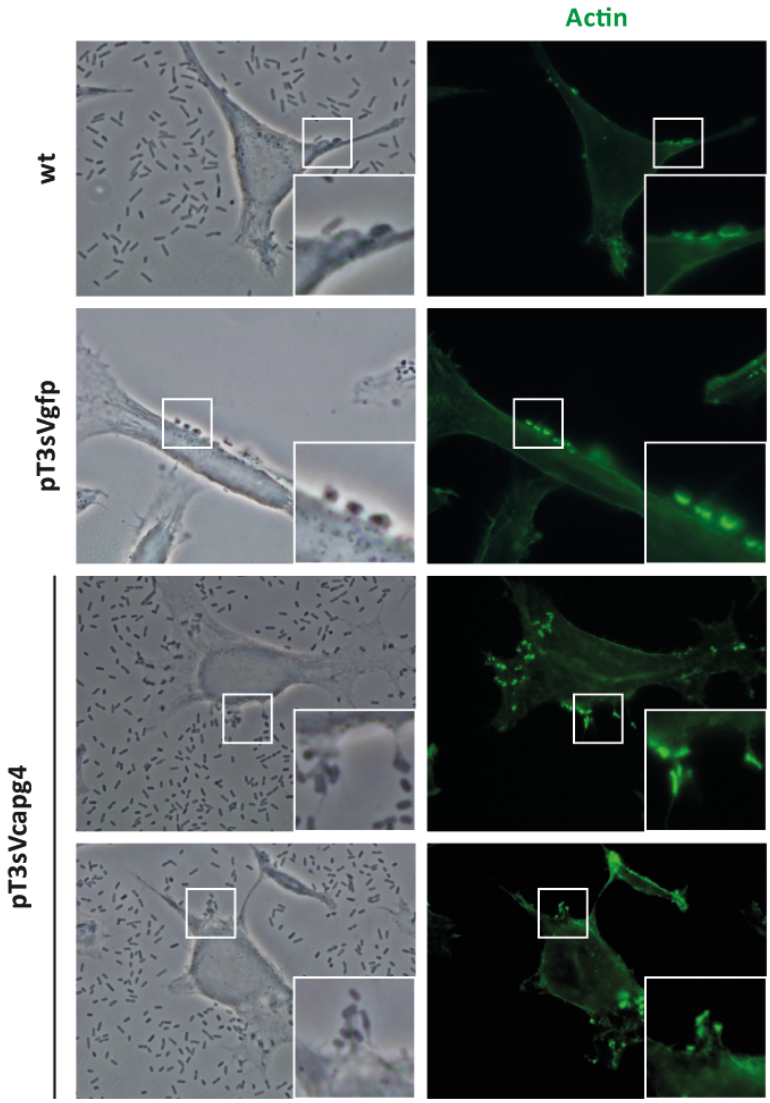


Figure 31. VcapG4 injected by EPEC bacteria affects the formation of actin pedestals. Representative microscopy images of *in vitro* cultured HeLa cells infected with wild type EPEC bacteria (wt) alone or encoding fusions of a T3 secretion signal to nanobodies against GFP (pT3sVgfp) or CapG (pT3sVcapG4), as indicated. The actin cytosckeleton was stained (in green) to reveal actin pedestals underneath the attached bacteria. Selected areas (white squares) of actin pedestals are shown at higher magnification to observe the projections of the plasma cell membrane.

2. Generation of a Synthetic Injector *E. coli* (SIEC) strain.

The T3SS of EPEC has been proved to be useful to inject proteins into the cytoplasm of eukaryotic cells. Nevertheless, EPEC is a pathogen that also tranlocates effectors that are directly related with the virulence of the strain into the cytoplasm of the infected cells. Therefore, we decided to generate a non-pathogenic *E. coli* strain with the ability to inject proteins through the T3SS. This strain would express all of the essential components needed for the assembly of functional injectisomes from the chromosome in a controlled manner. Moreover, this strain would also lack natural effectors present in the LEE island, and would not present any antibiotic resistance, since all the genetic determinants for the T3SS would be integrated into the genome.

2.1. Selection of the T3SS-coding genes.

The T3SS, or injectisome, is a macromolecular structure located in the bacterial envelope that has great complexity[42]. In EPEC, more than 20 proteins encoded in the LEE island participate in its assembly, including chaperones and secretion regulatory components. Our aim was to isolate the necessary genes for the assembly of the T3SS and integrate them in a non-pathogenic *E. coli* to generate the projected strain named Synthetic Injector *E. coli* (SIEC). To do that, we discarded all of the effectors, the transcriptional regulators and the natural promoters, selecting only the coding sequences of the structural genes, the chaperones and the translocators (Figure 32). The total genes selected summed up 27. We conserved the original order of the selected genes and the intragenic regions inside the genome to maintain any possible internal regulation. Thus, we designed 5 transcriptional units attending to the genome organisation of the LEE island and called them synthetic LEE (eLEE) operons: eLEE1, eLEE2, eLEE3, eLEE4 and eEscD. These eLEEs, with different lenghts are depicted in Table 10 with the genes present in each one.

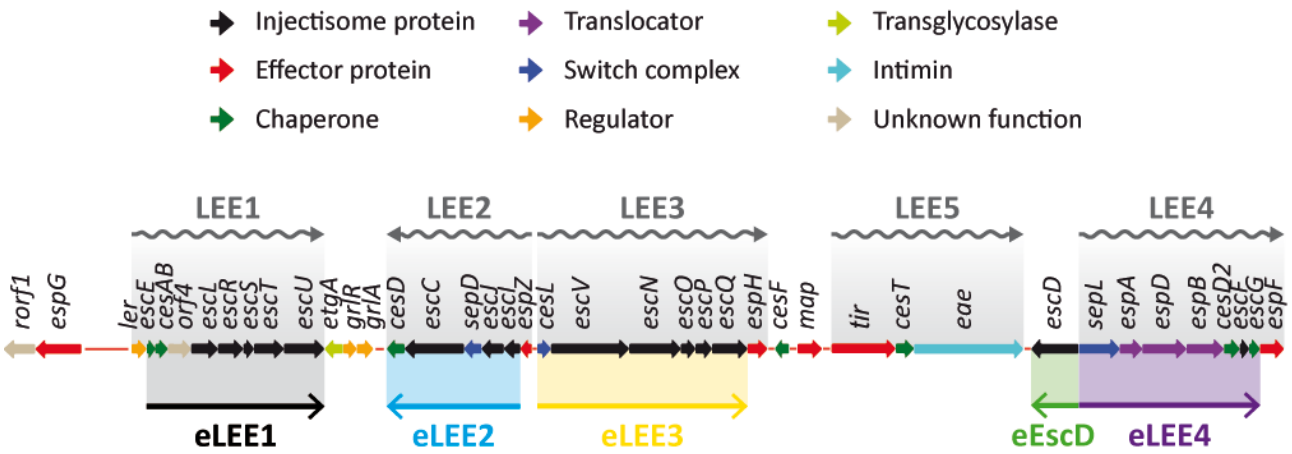


Figure 32. Genome organisation of the selected synthetic operons (eLEEs). The selected genes for the assemble of the T3SS couls be organised in five transcriptional units (eLEE1, eLEE2, eLEE3, eLEE4 and eEscD) that are depicted in different colours. The five main operons of the LEE island are coloured in gray.

Table 10. List of the genes present in the eLEE operons	
Engineered operon	**Genes**
eLEE1	escE, cesAB, orf4, escL,
eLEE2	escI, escJ, sepD, escC, cesD
eLEE3	cesL, escV, escN, escO, escP, escQ
eLEE4	sepL, espA, espD, espB, cesD2, escF, escG
eEscD	escD

2.2. Selection of the integration sites.

We selected the strain *E. coli* K-12 MG1655 lacking type 1 fimbriae (EcM1) as the parental strain to integrate the synthetic operons in the genome. We found attractive to integrate the synthetic operons in sites of the genome that correspond with genes that enhance the adhesive properties of *E. coli*, so to generate a SIEC strain engineered to have lower adhesion capacities. We selected some genes that have been identified to be related with adhesive properties of *E. coli*, as possible sites for recombination. They are depicted in Table 11.

Table 11. Selected integration sites for the integration of the eLEE operons.

Gene locus	Description	Reference
yfaL	Cryptic adhesin	Roux, *et al*., 2005[300]
yeeJ	Cryptic adhesin	Roux, *et al*., 2005[300]
yra	Cryptic fimbrial operon	Korea, *et al*., 2010[180]
yebT	*Vibrio* MAM7 adhesin homolog	Krachler *et al*., 2011[181]
yfc	Cryptic fimbrial operon	Korea, *et al*., 2010[180]
sfm	Cryptic fimbrial operon	Korea, *et al*., 2010[180]
flu	Antigen 43	Roux, *et al*., 2005[300]
mat	*E. coli* Common pilus	Pouttu *et al*., 2001[283]

First, we wanted to elucidate if EcM1 could express heterelogous proteins from the selected sites. To this end, we integrated the reporter gene GFP^{TCD} (green fluorescent protein enhanced for its production in bacteria)[60] under the control of the *tac* promoter in the different selected genomic sites using plasmid pG and following a strategy based on homology recombination that leaves no marks in the genome (see materials and methods). As a result, we generated eight individual strains that had substituted each target site for the GFP^{TCD} coding sequence. To analyse the expression of GFP^{TCD} in each strain we grew liquid cultures and induced them with IPTG. We tested two conditions for the induction: 3 h and an overnight induction. We subjected the cultures for cytometry analysis together with a control lacking GFP^{TCD} (Figure 33). All of the strains harbouring GFP^{TCD} emitted green fluorescence in both conditions. We observed that all of the populations expressed GFP^{TCD} at 3 h with a low variability inside the population. We also observed homogeneous mean levels of expression among the different strains, except for the expression level of GFP^{TCD} from the *yeeJ* site of integration - being much higher than from the others -, and from the *sfm* site - that had the lowest mean expression detected. These results were analogous in the overnight cultures, as the expression pattern of GFP^{TCD} in the different strains was very similar, despite the general higher signal observed, that may be due to the stability of the protein. In Figure 33C, it is represented the mean expression of each of the cultures. Given the low mean expression of GFP^{TCD} from the sfm site, we discarded it for the integration of the synthetic operons. So, we decided to select integration sites yeeJ, yebT, yra, yfaL and yfc. We reserved flu and mat integration sites for future modifications of the strain.

In a previous study on the levels of expression of the natural operons, it had been observed that the level of expression of the LEE2 operon is higher than operon LEE3[40], while in other study, it was observed that operon LEE4 is expressed at higher levels than operons LEE3 and LEE1[201]. We tried to maintain the relative expression hierarchy of the natural operons choosing the integration site for each operon according to the relative data obtained of the gfp expression from the different integration sites: yeeJ with LEE2, yebT with LEE4, yra with LEE3, yfaL with LEE1 and EscD with yfc.

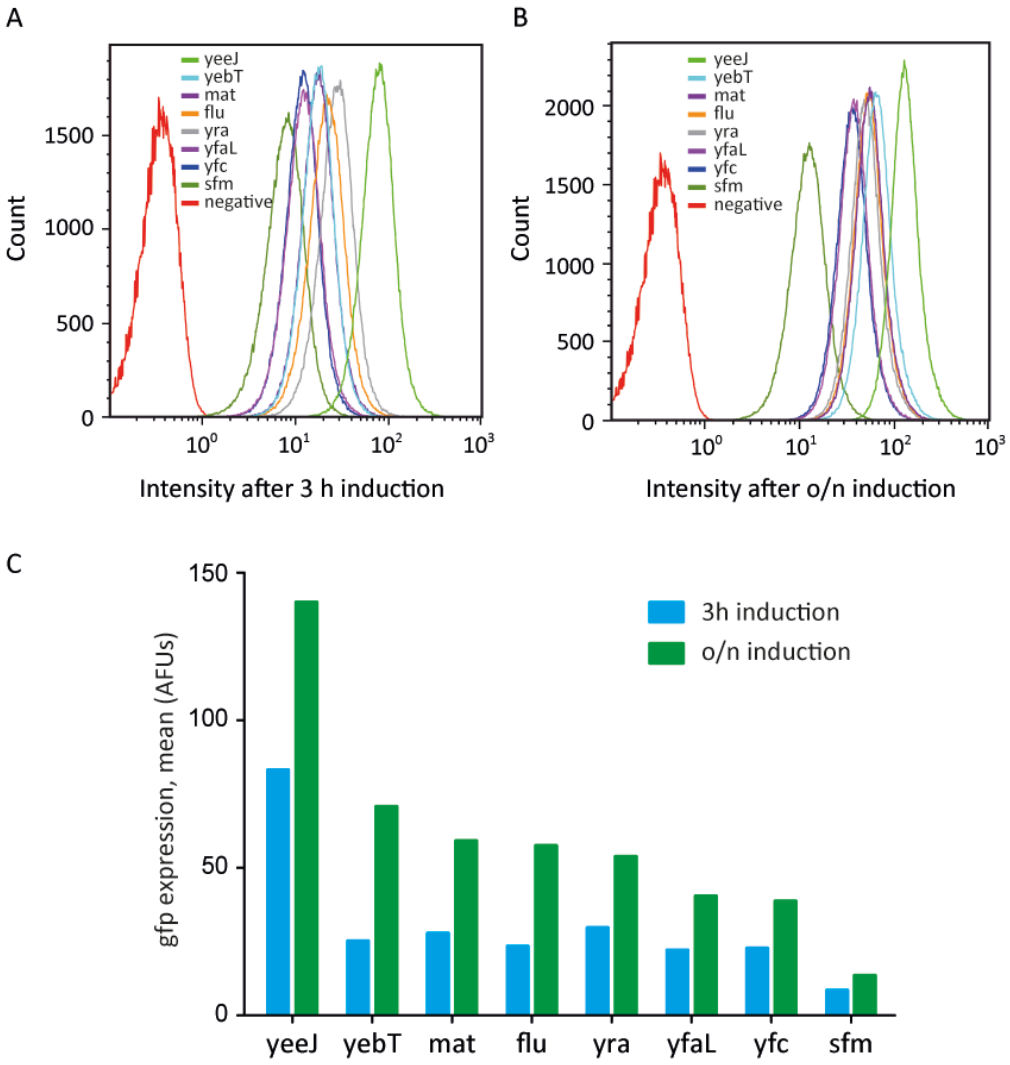


Figure 33. Cytometry analysis of the expression of GFP^{TCD} from the chromosome. GFP^{TCD} expression was induced during 3 (A) or 16 h (B) in EcM1 strains with the GFP^{TCD} coding sequence integrated in different sites of the genome. We also used a control that lacked GFP^{TCD}. C. The mean expression of GFP^{TCD} was plotted to compare the expression levels from the different sites of the genome.

2.3. Integration of the engineered LEE operons in the genome of *E. coli* MG1655.

To integrate the operons into the genome of EcM1, we cloned the homology regions of each integration site into pGETS. We amplified the five selected transcriptional units of EPEC by PCR and cloned them in

their respective pGETS plasmid pair under the regulation of the Ptac promoter adding a strong RBS after the promoter for the proper translation of that gene. For the cloning of the eLEE1 operon we had first included Ler, the master regulator of the expression of the LEE island, since Ler positively regulates the expression of the native operons[91]. However, we were not able to reach the construct with that configuration. Thus, we decided to clone the operon without promoter (eLEE1*) to prevent a possible toxicity derived from the leakage of the promoter, amplified by the high number of copies of the plasmid in the bacteria.

We integrated the operons in the genome in a double recombination event based on the lambda-red system described by Datsenko and Banner (see materials and methods). We decided to integrate the operons following the natural order of the T3SS assembly as much as possible. The process was done sequentially starting with the eLEE2 operon. We used the modified EcM1 that expresses GFP^{TCD} from the yeeJ site as the starting strain, which we had already generated. In this case, the integrative mutants lacked expression of GFP^{TCD}, so we did not require selection with ampicillin. The subsequent integrations, followed order eLEE3, eEscD, eLEE1* and eLEE4, and after each integration we transformed plasmid pCP20 for the removal of the integrated ampicillin resistance cassette. This plasmid expresses the flipase enzyme, which recombines the FRT sites. After that, we selected the clones sensitive to ampicillin and transformed the next integrative plasmid proceeding the same way until the last one. Once we had integrated all of the operons we obtained strain SIECΔp1. To integrate the promoter of the eLEE1 operon, we constructed a plasmid derived from the pGE, where the Ptac promoter was flanked by an homology region corresponding with the first gene of the operon (Ler) and the homology region used for the integration of the eLEE1 operon (yfaL). But we could not reach that construct either, given that the positive clones for the cloning presented mutations in the sequence of Ler. So, we decided to remove Ler from the final construct using the second gene, escE, as an homology region for the integration of the Ptac promoter. This construct presented no mutations in the sequence of EscE, indicating the expression of Ler from the plasmid must cause toxic effects in EcM1. We integrated this plasmid in SIECΔp1 and obtained the final strain, SIEC. Interestingly, using this strategy we also got strain SIECΔp1, which is unable to express the genes of the operon eLEE1 and is hence defective for the production of the T3S injectisomes.

3. Characterisation of SIEC

We had integrated all of the operons that harbour the selected proteins to assemble injectisomes on the recombinant strain that we called SIEC. We then dealt with the demonstration that SIEC expresses functional injectisomes that are able to secrete proteins to the culture medium. The injectisomes should also have a similar structural configuration as the needle complexes of EPEC.

3.1. Functionality of the injectisomes of SIEC

We grew SIEC and the parental strain EcM1 in liquid culture with IPTG to determine if the expression of the engineered operons could be hindering the growth of the SIEC strain (Figure 34). Both strains showed a similar growth rate. At 3 h, the strains were already in stationary phase, so we selected that timepoint to analyse the functionality of the T3SS by observing if the translocators (EspA, EspB and EspD) had been secreted to the supernatant. We also incubated the cultures 6 and 16 h to compare the possible accumulation of the translocators in the supernatant.

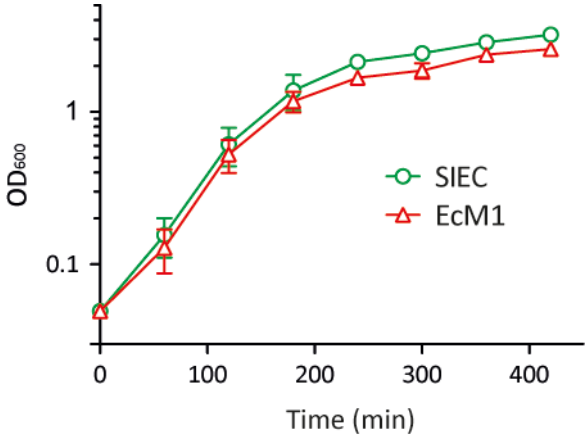


Figure 34. Growth curve of SIEC and EcM1. Both strains were grown in LB broth with IPTG and we measured the OD at 600 nm each 60 min.

We grew SIEC in liquid cultures with and without inducer and analysed the precipitated proteins from the supernatants by Coomassie staining in the selected timepoints (Figure 35). The secretion of the T3SS translocators was not detected in the supernatant of the uninduced cultures, while we observed bands of the corresponding sizes of EspA, EspB and EspD in all of the inducing conditions tested. This indicates that the T3SS expressed in SIEC is functional. We observed accumulation of these proteins with the time. However, in the overnight culture we observed additional bands, indicating possible lysis of the culture. Consequently, we decided to induce at 6 h herinafter. We also analyzed the supernatants by Western blot to detect EspA and EspB. Consistent with the data obtained from the Coomassie analysis, translocators EspA and EspB were present in the supernatant of the induced cultures. We also identified little secretion of EspA and EspB in the cultures without inducer, indicating some escape of the expression of the operon from the Ptac promoter.

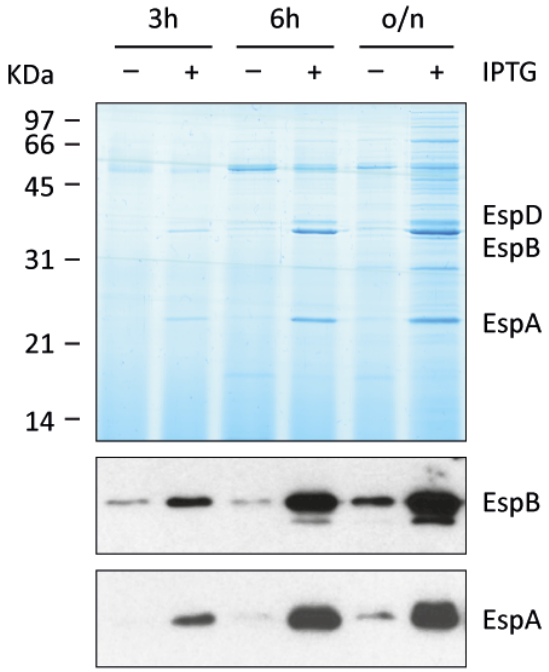


Figure 35. Secreted proteins of SIEC to the supernatant. The supernatants of SIEC cultures grown with or without IPTG during 3, 6 or 16 h were analysed by Coomassie staining. EspA and EspB tranlocators were detected by Western blot.

Then, we compared the expression of structural proteins of the T3SS in SIEC with the natural regulation of EPEC. As controls for the expression of these proteins we used EPEC*ΔescN*, uninduced SIEC, SIECΔp1 and the parental strain, EcM1. We grew the strains in the set conditions and analysed the bacterial lysates by Western blot (Figure 36B). EcM1 expressed none of the analysed proteins from the T3SS. We analysed two proteins present in the eLEE2 operon: the outer membrane secretin EscC and the inner ring forming protein EscJ. These two proteins had a higher expression in SIEC under the tested conditions compared to EPEC. The protein EscD, expressed alone in one operon, behaved the same way. In the case of EscN, expressed from eLEE3, the expression in SIEC and in EPEC was very similar but, interestingly, the size of the protein in SIEC was smaller than in EPEC, indicating that there might be some posttranscriptional modification in EPEC that is not ocurring in SIEC. As expected, EPEC*ΔescN* did not expresse this protein. The translocators EspB and EspA, expressed from the eLEE4, were also detected in the cell extract of SIEC with a slight increase of the expression level compared to EPEC. As a loading control we analysed the expression of the cytosolic chaperon GroEL, a constitutively expressed protein of *E. coli*. Comparing the expression with uninduced SIEC we detected all of the proteins due to the leakage of the promoter. Surprisingly, EspA and the ATPase escN had a more conspicuous reduced signal, indicating that the level of expression of these proteins may be determinant for the secretion. In the case of SIECΔp1 we observed the same levels of expression as in SIEC in most of the proteins but EspA, were there was an unexpected reduction of the expression. It is interesting that the reduction of the expression of EspA was also observed in EPEC*ΔescN*.

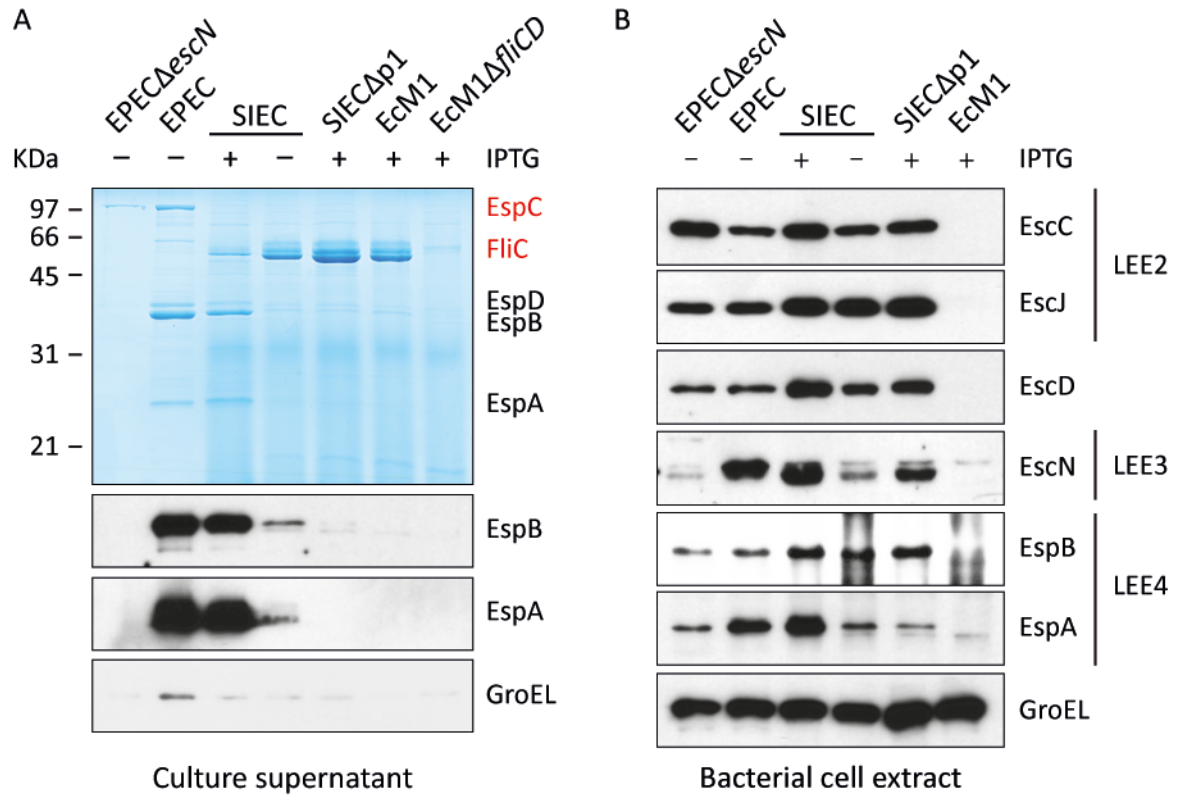


Figure 36. Expression and secretion of proteins of the T3SS. EPEC*ΔescN*, EPEC, SIEC, SIECΔp1, EcM1 and EcM1*ΔfliCD* were grown with or without the presence of IPTG in LB broth. A. Coomassie staining and Western blot of the culture supernatants. EspA, EspB and GroEL were detected with specific antibodies. B. Western blot of the bacterial cell extracts detecting proteins of the T3SS and GroEL as a loading control.

The proteins present in the culture supernatants were analysed by Coomassie staining and Western blot (Figure 36A). In the Coomassie staining, EPEC*ΔescN* and EPEC presented a 100 KDa band that corresponds with the T3SS-independent autotranslocator EspC, but only EPEC showed secretion of EspA, EspB and EspD. The corresponding bands of the translocators also appeared in SIEC, but only if grown in the presence of IPTG, as shown above. SIECΔp1 was also defective for secretion and presented the same band pattern as the parental strain EcM1. Moreover, SIECΔp1, EcM1 and the uninduced culture of SIEC presented an extra band of about 55 KDa. Given that EcM1 is a hyperflagellated strain we thought that the band might correspond with the flagellin (FliC). We analyzed this using a EcM1-derived mutant strain in FliC (EcM1*ΔfliCD*). This mutant did not show this band, so we confirmed our hypothesis. Interestingly, FliC was secreted at lower levels when the T3SS was induced in SIEC, if we compared the band with the uninduced condition.

When we analysed the supernatants by Western blot to confirm the identity of the proteins of the T3SS, we observed secretion of EspA and EspB in EPEC and SIEC. As described above, SIEC secreted a little quantity of the translocators in the non-inducing condition due to the leakage of the Ptac promoter. On the other hand, we detected no secretion of EspA and very little of EspB in SIECΔp1, indicating that the mutation in the promoter of the eLEE1 totally abrogates the secretion by the T3SS. We also tested the signal of GroEL in

the supernatant to check if the presence of the proteins in the supernatant was due to cellular lysis. We detected non-significant levels of GroEL in all cases, indicating that the translocators were present in the supernatant due to the activity of the T3SS.

Taking these results altogether, we demonstrated that SIEC can assemble functional T3SS injectisomes capable of secreting translocator proteins in a controlled manner. We also compared the secretion levels produced by SIEC with the yield reached by EcM1 harbouring the pCVD462 cosmid, which carries the whole LEE island[234]. We grew SIEC and EcM1pCVD462 in LB liquid medium under inducing conditions and analysed the secreted proteins of the supernatant by Coomassie (Figure 37). The proteins of the supernatant of EcM1pCVD462 lacked the bands corresponding with the translocators. We could not either detect EspA by Western blot and the secretion of EspB produced by this strain was radically lower than the one produced by SIEC. On the other hand, EcM1pCVD462 only presented the band corresponding with FliC. This indicates that the T3 secretion by SIEC is much more efficient than the secretion of EcM1 harbouring the cosmid pCVD462, with the improvent of having a controlled expression and lacking all of the effectors and the internal regulators.

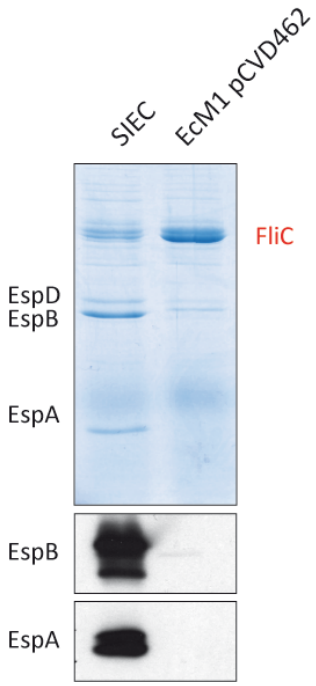


Figure 37. Secreted proteins of SIEC and EcM1 harbouring a cosmid encoding the LEE island. The supernatants of SIEC and EcM1pCVD462 cultures were analysed by Coomassie staining and Western blot detecting EspA and EspB.

3.2. Relation of the expression of the T3SS and the flagellum

The flagellum and the T3SS share structural similarities. Derived from the observation that the expression of the T3SS in SIEC affects to the secretion of the flagellin, we wanted to elucidate if this interference was uni- or bidirectional, that is, if the flagellum could be affecting to the T3SS, hindering the secretion of the

translocators. To address this concern, we performed a swimming motility assay on LB 0.3% agar plates growing individual colonies of SIEC, SIECΔp1, EcM1 and EcM1*ΔfliCD* during 8 h (Figure 38). We represented the relative radius length of the each colony in relation with the radius of EcM1. SIEC showed a significant reduction in its capacity to swim to a 55%. This reduction of the flagellar activity can be attributed to the expression of the T3SS proteins, given that the SIECΔp1 restored the motility showed by EcM1 to a 92%. As expected, EcM1*ΔfliCD* showed no motility due to the lack of flagellin.

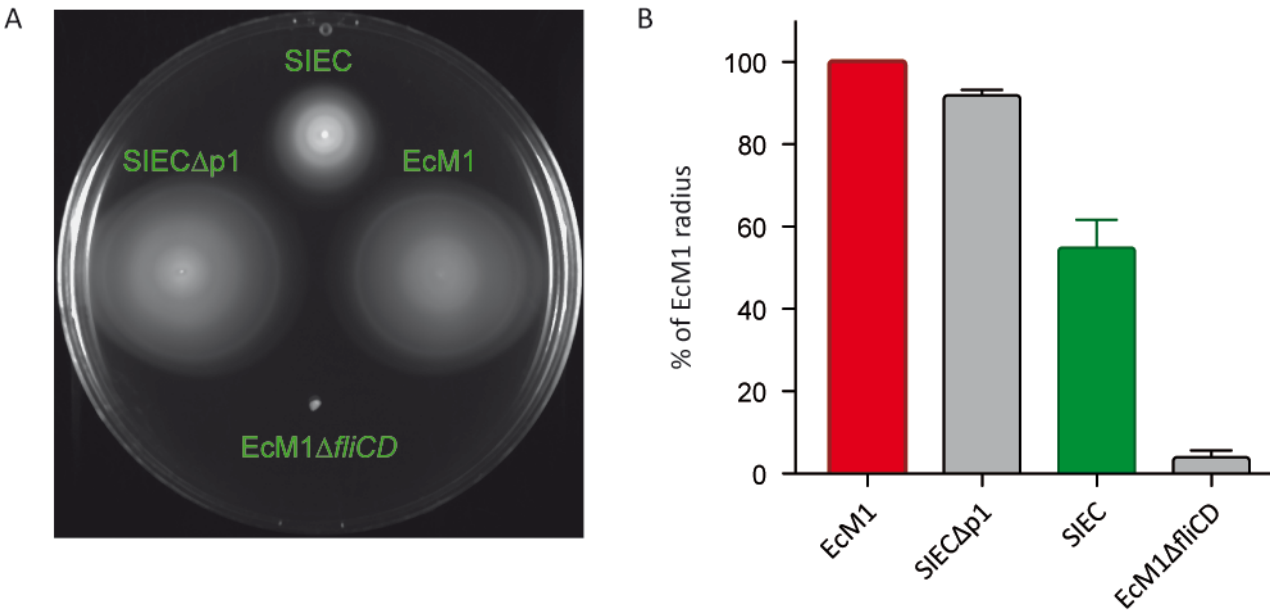


Figure 38. Swimming motility assay. A. SIEC, SIECΔp1, Ecm1 and EcM1*ΔfliCD* were inoculated in soft LB-agar plates. B. Representation of the mean radius with SEM of the obtained colonies in relation with the radius of EcM1 in three independent experiments.

Given that all of the proteins of the T3SS were being expressed in SIECΔp1 but the ones from the eLEE1 operon, we thought that those proteins might be affecting to the polimerisation of the flagellum. To elucidate this, we integrated the eLEE1 operon along with its promoter into EcM1 using the same strategy as above. The resulting strain (EcM1-eLEE1) expresses the proteins of the eLEE1 operon upon induction with IPTG. We analysed the proteins present in the supernatant of an induced culture of this strain by Coomassie staining (Figure 39C). Interestingly, EcM1-eLEE1 showed a reduction in the secretion of FliC to the supernatant and lacked the translocators. This indicates that one or some proteins of the eLEE1 might be hindering in some way the secretion of the flagellin to the culture medium. We confirmed this observation in a swimming motility assay on LB 0.3% agar plates (Figure 39A). The motility of EcM1-eLEE1 on these plates was significantly reduced to a 66%, while SIEC showed a significant reduction to a 52% (Figure 39B). Taking these results altogether, we demonstrated that proteins of the eLEE1 operon were interfering with the machinery of the flagellum assembly.

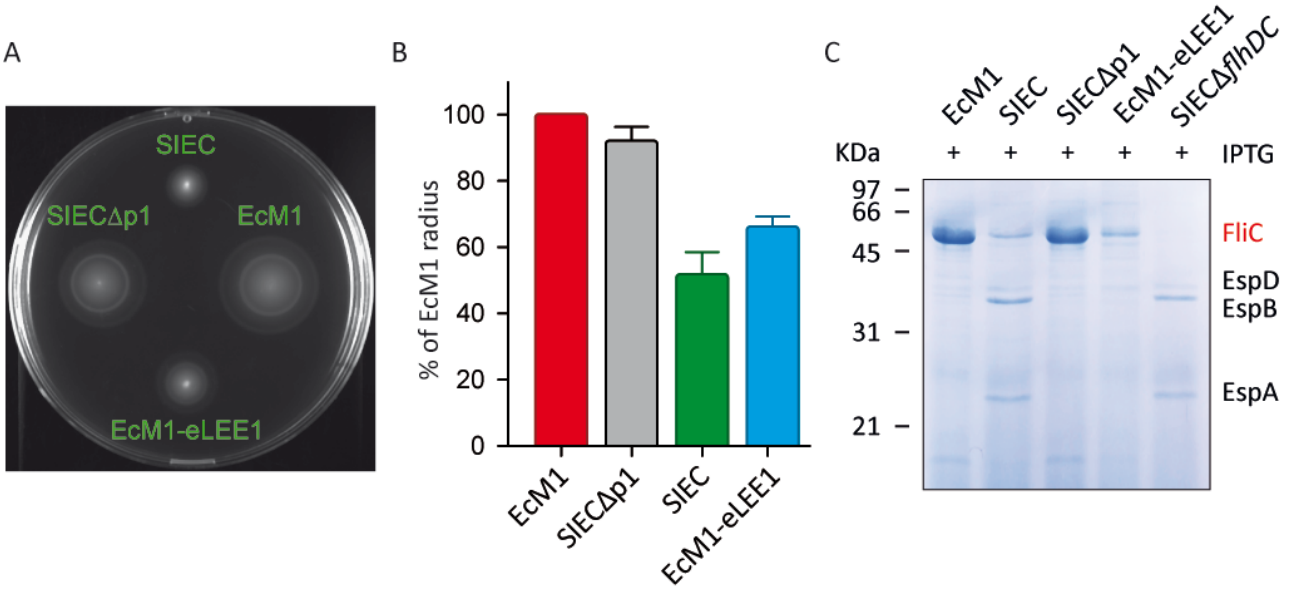


Figure 39. Interference between the T3SS and the flagellum. A. Swimming motility assay of SIEC, SIECΔp1, EcM1 and EcM1-eLEE1. These strains were inoculated in soft LB-agar plates. B. Representation of the mean radius with SEM of the obtained colonies in relation with the radius of EcM1 in three independent experiments. C. Coomassie staining of the supernatants of EcM1, SIEC, SIECΔp1, ECM1-eLEE1 and SIEC*ΔflhDC* grown in inducing conditions.

The similarities of these systems led us to speculate that the expression of the flagellum might also be affecting to the yields of secretion observed in SIEC. To elucidate this, we deleted the operon *flhDC* of the genome of EcM1 by homologous recombination using the plasmid pGE. These two genes are organised in an operon and are considered the master regulators of the expression of all the flagellar proteins[376]. By mutating them, we wanted to prevent any possible interference of the flagellar proteins with the T3SS expression. We mutated these genes in SIEC generating a SIEC*ΔflhDC* strain and analyzed the secreted proteins of an induced culture by Coomassie staining (Figure 39C). SIEC*ΔflhDC* secreted no flagellin as expected, but the level of secretion of the tranlocators remained very similar to the secretion observed in SIEC. These results indicate that the expression of the T3SS affects to the flagellar motility, but not eitherwise.

3.3. Electron microscopy analysis of the injectisomes of SIEC

Once we had demonstrated that T3SS is functional when expressed in SIEC, we moved forward to visualize the injectisomes complexes on the bacterial membrane and compare their structure with the original injectisomes of EPEC. The EspA filaments are up to 700 nm long and can be observed on the surface of T3SS-expressing strains. We negatively stained the bacterial cells of EPEC, EPEC*ΔescN*, SIEC and SIEC*ΔflhDC* with uranyl acetate for the observation at the transmission electron microscope (Figure 40). EPEC showed on its surface filamentous organelles that matched with previous observations of the EspA filaments of the injectisome[69], but no such filaments were seen on T3SS-defective EPEC*ΔescN*. In the surface of SIEC we observed structures highly similar to the ones observed on EPEC in terms of width and length, which could be EspA filaments. SIEC*ΔflhDC* allowed us to assess whether these filaments corresponded with the

flagellum. Interestingly, SIEC presented on its membrane only these filaments, indicating that the strategy of removing fimbriae and other adhesins resulted in a membrane with less attaching capacities but the adhesion conferred by the injectisomes.

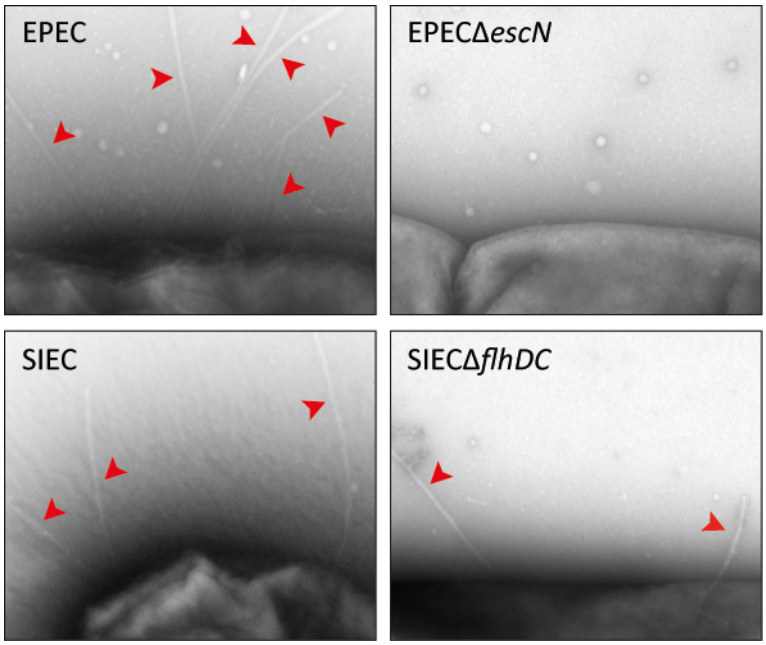


Figure 40. Transmission electron microscopy of EPEC, EPEC*ΔescN*, SIEC and SIEC*ΔflhDC*. Bacteria were negatively stained for the observation of the injectisomes, that are pointed out with red arrowheads.

To confirm these observations, we decided to purify the needle complexes from the bacterial membrane of SIEC and EPEC in order to observe the supramolecular structure of the T3SS. We centrifuged induced grown liquid cultures to get the pellet, which was lysed with a soft detergent. The cell fraction containing the injectisomes was isolated and subjected to a CsCl density gradient centrifugation. We collected 11 fractions from the top to the bottom of the gradient. At the described purification steps we took representative fractions that were analysed together with the aliquots of the gradient by Western blot to detect EspA (Figure 41). We identified EspA both in EPEC and SIEC accumulated in the cell pellet (M1) and in the injectisome-enriched fraction (M2). In the fractions obtained after the centrifugation in the CsCl gradient, we detected EspA in the first aliquots - where cellular debrises are abundant – and in fraction 10 and 11. We selected fraction 10 for the observation at the transmission electron microscope by negative staining (Figure 42). These fractions presented sheath-like structures with a basal body at one end, highly similar with injectisomes previously described[325]. We selected 10 particles of each preparation and aligned them to compare the general structure. The needle structures showed different lenghts, with sizes ranging between 50 and 400 nm in SIEC and EPEC. The structures were thus highly similar, tough we observed slightly thickened filaments in the identified injectisomes of SIEC. Hence, we demonstrated that SIEC is capable of assembling injectisomes heterologously on its membrane and that they share a conserved structure with the EPEC needle complexes. We have also demonstrated that the proteins that we integrated in this study in the genome of SIEC are sufficient to form the structure of the injectisome on the membrane.

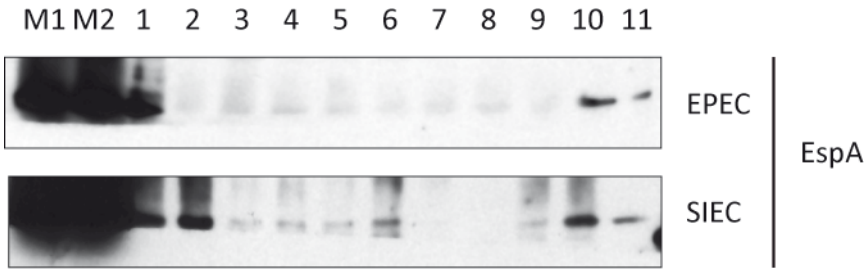


Figure 41. Isolation of injectisomes by cell fractionation. The membrane fraction of EPEC and SIEC lysed cultures was subjected to ultracentrifugation in a CsCl gradient. The obtained fractions (1 to 11) were analysed by Western blot together with the cell pellet (M1) and the injectisome-enriched cell fraction (M2) to detect EspA.

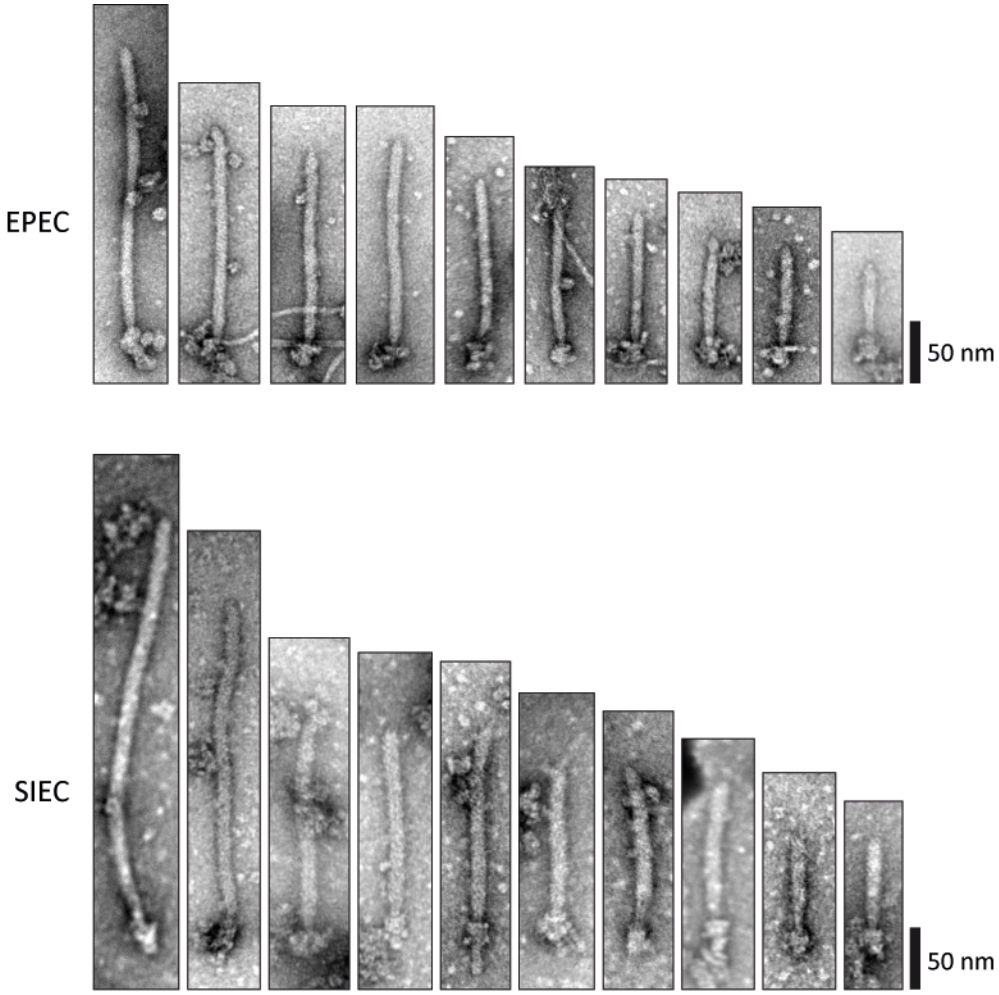


Figure 42. Isolated injectisomes of EPEC and SIEC. The purified injectisomes were negatively stained for the observation at the transmission electron microscope. The black bars indicate the scale.

4. Injection of proteins into mammalian cells using SIEC

We had demonstrated that the injectisomes expressed in SIEC are capable of secreting translocators to the culture supernatants, but we wanted to take a step further and determine if SIEC was capable of forming pores on eukarytotic cells and inject proteins directly to their cytoplasm.

4.1. Injection of Tir to form actin pedestals.

As a first approach, we measured the osmotic lysis caused in red blood cells by the EspB/D translocation pore upon contact with their membrane. This assay has been previously used to determine the formation of functional injectisomes by measuring the liberation of hemoglobin derived from the cell lysis. We induced the assembly of the T3SS in EPEC, EPEC*ΔescN*, SIEC and SIECΔp1 in liquid cultures and incubated them with a solution of erythrocytes. The supernatants were used to measure the optical density at 450 nm. Considering that EPEC causes a 100% of hemolysis, we calculated the lysis caused by the other strains related to EPEC. We also used EcM1 as a blank to measure the spontaneous liberation of hemoglobin and substracted it from the data obtained with the other strains (Figure 43). EPEC*ΔescN* displayed a reduction of the lytic activity to a 10% and SIECΔp1 to less than a 5%. On the other hand, SIEC reached a 70% of EPEC hemolysis, indicating that the translocators secreted by SIEC are functional and capable to form the translocating pores on the membrane of the infected cells, which are needed for the injection of proteins.

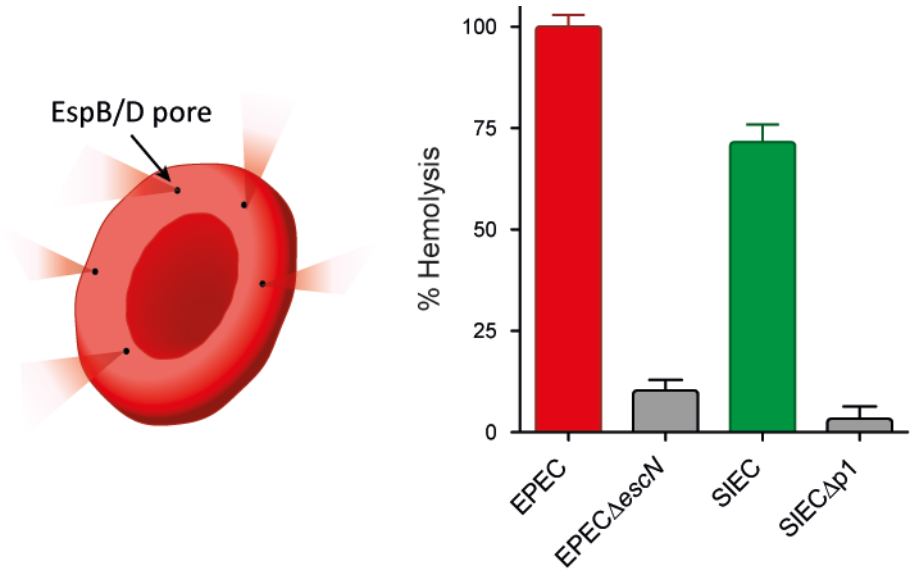


Figure 43. Hemolytic capabilities of EPEC, EPEC*ΔescN*, SIEC and SIECΔp1. A. The EspB/D complex forms pores on the membrane of erythrocytes causing the release of hemoglobin. B. Hemoglobin released into the supernatant was measured by determining OD at 450 nm after incubation of the strains with rabbit RBCs. The mean data and the SEM of three independent experiments were represented in comparison with EPEC and substracting the data obtained with EcM1.

Moving forward, we tested the ability of SIEC to inject a natural effector of EPEC to eukaryotic cells and whether it is functional in the cytoplasm of the cells. We selected Tir as a model effector for the translocation as it triggers the formation of actin-rich pedestals when it is injected to the cytoplasm through the T3SS. To this end, we cloned the LEE5 operon of the LEE island of EPEC - which encodes the sequences of Tir, Intimin and the Tir specific chaperone CesT - into plasmid pGETS. Following the same strategy used for the integration of the previous operons, we integrated the sequence into the *flu* sites of

the genome of SIEC, obtaining strain SIEC-eLEE5. We also integrated it into the T3SS-defective strain SIECΔp1, obtaining strain SIECΔp1-eLEE5.

We analysed the expression of Intimin and Tir in the bacterial cell extracts of induced cultures of SIEC-eLEE5 and SIECΔp1-eLEE5 by Western blot, comparing them with the expression in EPEC and EPEC*ΔescN* (Figure 44). Tir was expressed in all of the strains, though EPEC and SIEC-eLEE5 showed higher expression. However, the expression levels of Intimin were lower in the SIEC-derived strains in comparison with EPEC and EPEC*ΔescN*. This indicates that the expression of the proteins of the LEE5 operon of EPEC might have additional internal regulatory sequences apart from the promoter. We also analysed the T3 secretion to the supernatant in a Coomassie staining (Figure 44). The translocators were present in SIEC-eLEE5 but not in SIECΔp1-eLEE5, indicating that the expression of the eLEE5 encoded proteins does not affect the activity of the synthetic T3S injectisome. We also performed a Western blot analysis of the supernatants to detect the secretion of Tir. We observed that SIEC-eLEE5 also secretes Tir to the culture medium. In SIECΔp1-eLEE5 we also detected little secretion of Tir, which may be due to some leakage derived from the overexpression of the eLEE5 proteins, as it happened with EspB in the supernatant of SIEC.

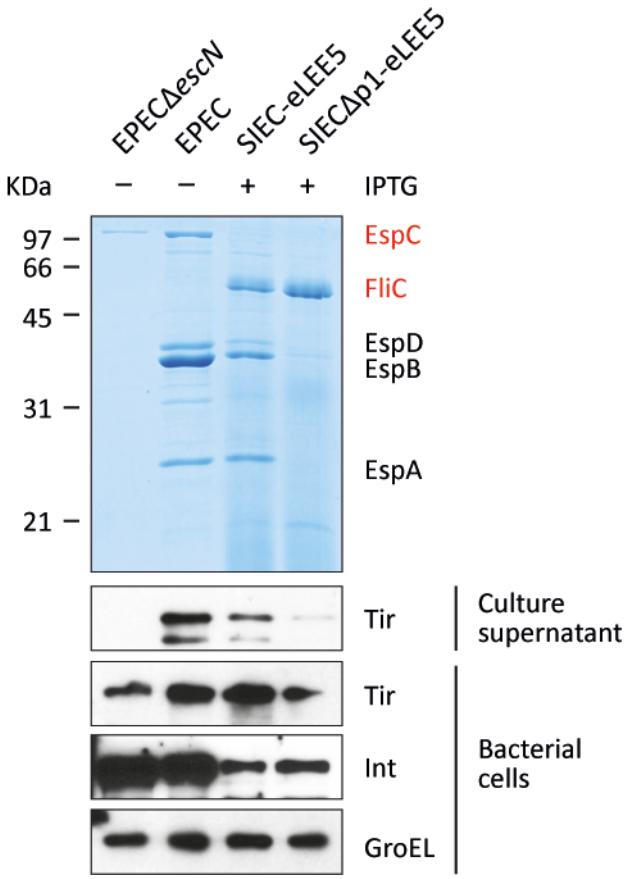


Figure 44. Expression and secretion of T3SS proteins by SIEC-eLEE5. The supernatants of EPEC*ΔescN*, EPEC, SIEC-eLEE5 and SIECΔp1-eLEE5 were analysed by Coomassie staining and the secretion of Tir by Western blot. The expression of Int and Tir was detected in the bacterial lysate by Western blot. GroEL was used as a loading control.

Next, we tested if SIEC-eLEE5 was able to inject of Tir to HeLa cells. We transformed SIEC-eLEE5 and SIECΔp1-eLEE5 with the GFP-expressing plasmid pGEN22 for the detection by green fluorescence. To induce the T3SS in SIEC-eLEE5 and SIECΔp1-eLEE5 we grew them in LB with IPTG for 150 min and then incubated them with the HeLa cells for another 180 min. EPEC and EPEC*ΔescN* were stained with an anti-Intimin antibody (Figure 45). Very interestingly, SIEC-eLEE5 adhered to the cells surface and we observed actin accumulation beneath the bacteria, which indicates that SIEC-eLEE5 is able to inject functional Tir to the cells cytoplasm, as lead to the formation of actin pedestals at the infection interface. This attachment is mediated by the activity of the T3SS, given that we SIECΔp1-eLEE5 is unable to bind the HeLa cells.

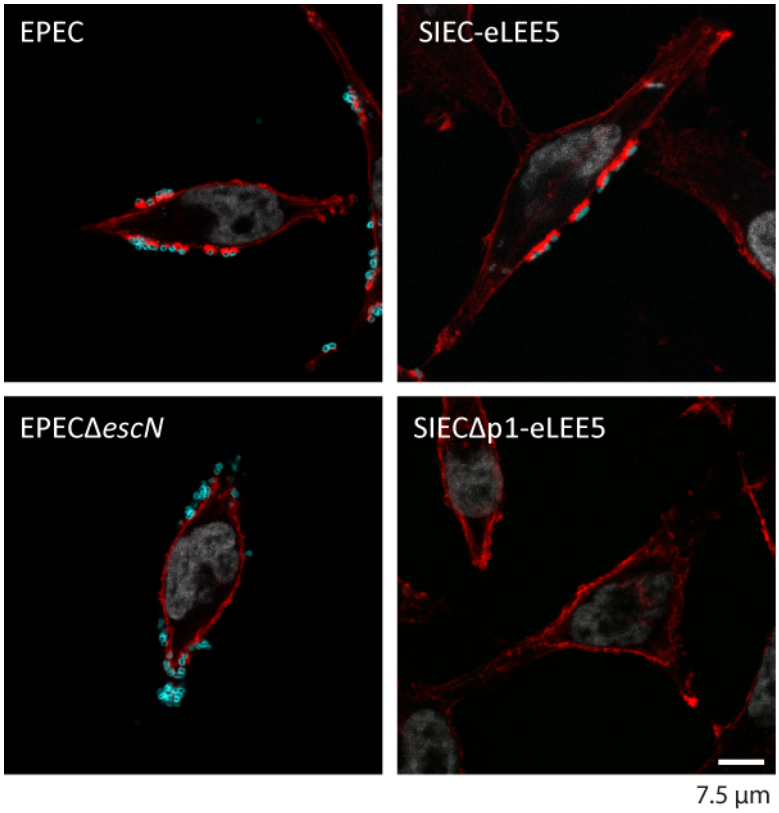


Figure 45. Infection of HeLa cells with SIEC-eLEE5. HeLa cells were infected with EPEC (upper-left panel), EPEC*ΔescN* (bottom-left panel), SIEC-eLEE5 (upper-right panel) and SIECΔp1-eLEE5 (bottom-right panel) to test the formation of actin pedestals beneath the bacteria. The actin cytoskeleton was stained in red. In cyan are depicted the bacteria, that whether expressed GFP (SIEC strains) or were stained with anti-Int (EPEC strains).

4.2. Internalisation of SIEC-eLEE5 after long periods of incubation.

EPEC presents a specific regulation of the injection of its effectors to the infected cells[24]. However SIEC-eLEE5 injects Tir continuously due to the artificial induction with IPTG. Also, it only injects Tir, as SIEC-eLEE5 lacks all of the other effectors found in EPEC. Thus, we wanted to analyse the effects of Tir after longer infection times on HeLa cells. We incubated SIEC-eLEE5 and SIECΔp1-eLEE5 harbouring pGEN22 with the cells prolonging the infection up to 240 min and stained actin for the observation at the confocal microscope (Figure 46). We detected the formation of more conspicuous actin pedestals and some bacteria

were almost completely surrounded by actin. We expected this effect, due to the lack of regulation of the injection of Tir. But, strikingly, many cells presented bacteria inside their cytoplasm, indicating that SIEC-eLEE5 had been internalysed during the infection process. To the contrary, we observed no binding or internalisation of SIECΔp1-eLEE5. We hypothesized that, after the formation of the pedestals, the Tir-derived response was also providing some sort of additional signal for the engulfing of the bacteria in between 180 and 240 min of infection.

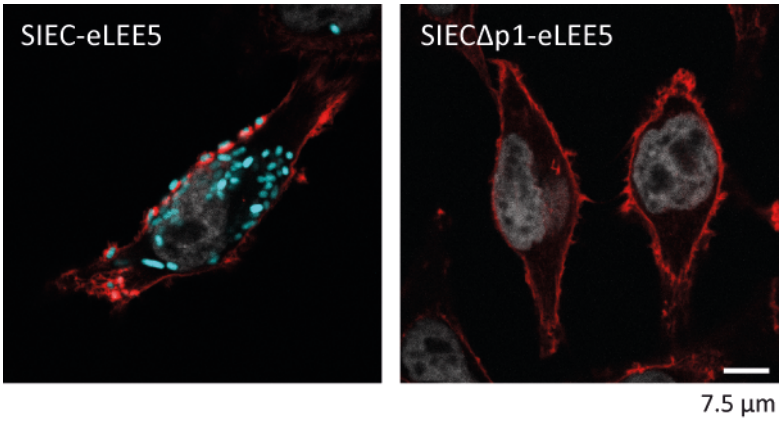


Figure 46. Infection of HeLa cells with SIEC-eLEE5 during 240 min. SIEC-eLEE5 (left) of SIECΔp1-eLEE5 (right) transformed with pGEN22 to express GFP (in cyan) infected HeLa cells during 240 min. The actin cytoskeleton was stained in red.

To analyse this, we decided to follow the infection process of SIEC-eLEE5 at the confocal microscope. We transformed the bacteria with pGEN22 for green flourescence and transfected the HeLa cells with a plasmid harbouring LifeAct-RFP, a peptide fused to the red flourescent protein[293]. We induced the expression of the T3SS of SIEC-eLEE5pGEN22 during 150 min in LB broth and then incubated the transfected HeLa cells with the bacteria for 180 min. We then started to follow the emission of green and red fluorescence at the confocal microscope for another 60 min. We selected already infected cells with actin pedestals and took photos each 30 seconds of different planes of the cells (Figure 47, Video 1). Very interestingly, the time-lapse video showed that some bacteria, after forming the actin pedestal, passed through the cellular membrane and were internalised, as we had previously observed. It took ca. 15 min for the internalisation after the first contact. This effect has not been described in longer infection times in EPEC, as it remains an extracellular pathogen, so we hypothesize that EPEC may be regulating actin polimerisation at some extent to avoid internalisation.

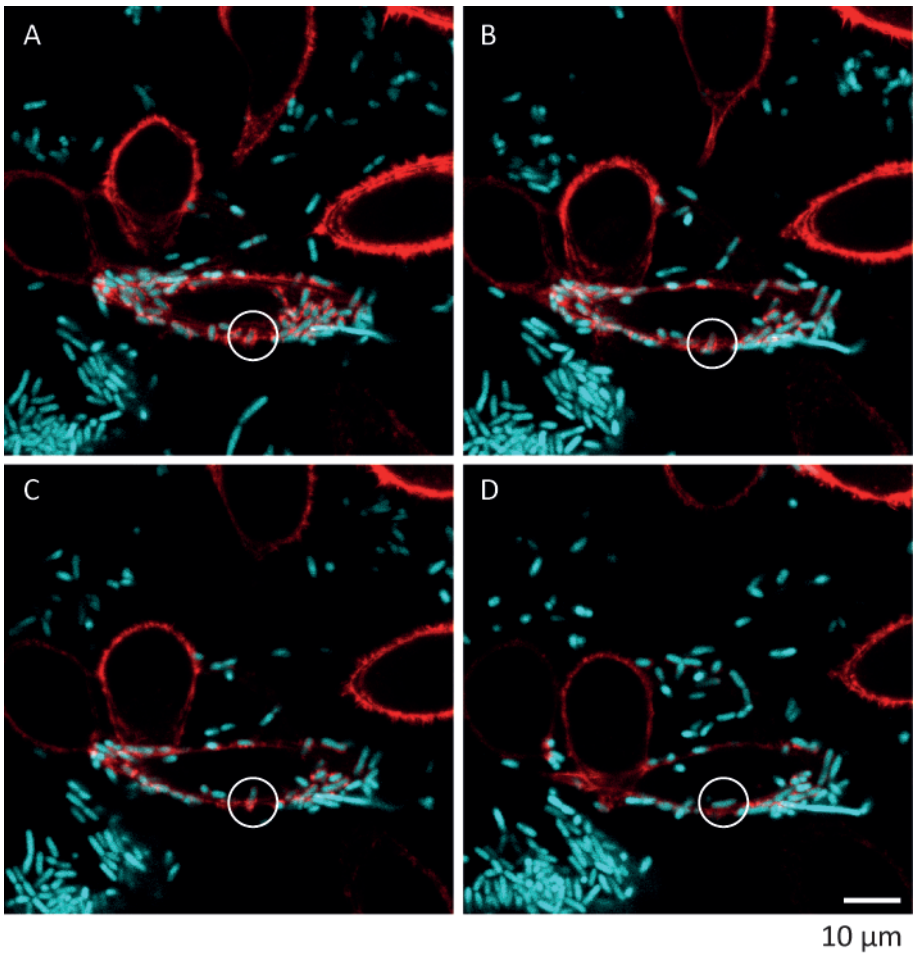


Figure 47. Internalisation of SIEC-eLEE5. SIEC-eLEE5 transformed with pGEN22 (in cyan) infected HeLa cells transfected with LifeAct-RFP (in red) during 240 min and a video was recorded by confocal time-lapse microscopy during 30 min within the last 60 min of infection. A-D show different moments of the process. The internalisation of one bacterium is highlighted Inside the white circle.

DISCUSSION

Hly secretion system for the selection of high afinity V_{HH}s from enriched libraries

Enterohaemorrhagic *E. coli* (EHEC) causes severe diseases such as bloody diarrhoea and HUS in humans due to the consumption of contaminated food or water[275]. The use of antibiotics is not recommended to treat these pathologies, because EHEC responds overproducing Stx[407], leading to an exacerbation of the infection; so the current treatment is based on fluid replacement and supportive care[238]. But the increasing knowledge on the virulence factors and the infection mechanisms has contributed to the development of new strategies to prevent disease[264]. To date, three major strategies are being developed to reduce the virulence of EHEC and the Stx production: inhibition of quorum sensing[288], specific degradation of the O157 LPS using bacteriocins[296] and inhibition of the binding of Stx to Gb3 with antibodies[243,246] or Stx-specific ligands[192].

In this work, we tested the possibility of generating specific V_{HH} antibodies to target proteins of the T3SS of EHEC as an alternative approach. EspA is the filament of the T3SS of A/E pathogens, and is thought to be participating in the initial contact of EHEC with the infected cells[327] and the injection of effectors to their cytoplasm. On the other hand, Int and Tir interact after the initial adhesion to promote the intimate attachment of the bacteria[69] by subversing the actin cytoskeleton[194]. Given that Tir is the receptor of Int, specific domains of these proteins (Int280 and TirM) participate in the interaction between the two proteins. The roles of Int, Tir and EspA are essential for the infection, what makes them good targets for their specific inhibition with antibodies. We used immune V_{HH} libraries for the selection attending to two aspects: V_{HH}s can be easily selected and produced in bacteria[98] and recognise epitopes that cannot be accessed with other antibody types[250]. For the enrichment of the generated immune libraries, we used Phage display, which is a highly reliable high-throughput method[187]. But Phabs do not give an idea of the affinity of the V_{HH}s, since the exposure of the V_{HH}s on the surface is multivalent, and individual positive binders have to be sequenced and then purified for their characterisation[178]. Here we made use of the HlyA tripartite secretion system (Figure 6) for the direct secretion of the V_{HH}s to the medium (Figure 12) as a method for selection. This system has been used for the secretion of model recombinant antibodies[106,323] or peptides until the date, but we determined that a wide repertoire of V_{HH}s can be secreted. We used the supernatants from cultures of isolated clones for the screening of V_{HH}s against the corresponding antigens and determined that the variability of clones was the same as using the Phage display system for selection (Table 9). We even identified clones not detected by Phage display, thus indicating that the HlyA fusions are a suitable tool for the selection of V_{HH}s and that the system supports a wide repertoire of sequence variability for secretion. We found four groups of sequences coding V_{HH}s against Int280 (A1, B7, B10, C1), six groups in the case of V_{HH}s against TirM (D4, E1, E4, F1, F2 and G10), and three for V_{HH}s against EspA (C1, C7, E6) and selected a representative V_{HH} clone for each group. This selection strategy also allowed us to directly relate the affinity of the clones to the levels of secretion through the HlyA secretion system (Figure

13), thus allowing the selection of those V_{HH}s with higher secretion yields and affinity. The HlyA secretion system also allows the direct use of the V_{HH}s, since bacteria do not lyse and the V_{HH}s fused to the HlyA secretion signal are secreted into the supernatant, where they are accumulated and from which they can be concentrated. The secretion minimizes the effect of intracellular or membrane proteases and could be applied for the production of antibodies in continuous reactors. Besides, the V_{HH}s can be easily purified using His-tag affinity purification from the culture supernatants in a single step. The selected V_{HH}s against EspA (EC7), TirM (TD4) and Int280 (IB10) had similar purification yields (1-4 µg/ml) as what it has been observed in previous studies[116,323] and high affinity against their respective antigens (Figure 14).

Identification of TD4 as an inhibiting agent against the infection of EHEC

TD4 and IB10 were capable of displacing the Int:Tir interaction *in vitro* (Figure 15), leaving open the possibility of their use as blocking agents against the infection of EHEC. The binding of these clones must be of higher affinity or stability than the natural binders. However, in infection assays on HeLa cell cultures (Figure 16), neither EC7 nor IB10 inhibited the formation of actin pedestals beneath the infecting bacteria. Since Int is an adhesin that covers all of the surface of EHEC when the transcription of the LEE PAI is induced, the inhibitory activity of IB10 could be masked, and a much higher concentration of the V_{HH} would be necessary to see an effect on the infection. For the same reason, EC7 might have not exhibited any protection, given that EspA multimerizes forming the filament of the injectisome. Interestingly, TD4 did show interference with the formation of actin pedestals (Figure 16). Tir functions as a receptor and is only exposed on the host cell surface when it has been already translocated[171]. Thus, the target of the V_{HH} is limited to the cells that are being attacked. As a result, the concentration of the available molecules to be recognised is broadly reduced, what may explain that the concentration tested for TD4 (1 µM) is enough to see an effect on the formation of actin pedestals.

Staining of Tir after the infection showed that TD4 hindered the formation of pedestals by preventing the characteristic Tir clustering produced at the interface of infection[357] (Figure 17), thereby hindering the intimate attachment of EHEC. This effect is maintained in long infection times (6 h) (Figure 18), what indicates that the V_{HH} remains bound to Tir with a low dissociation rate. This hypothesis was confirmed using SPR, which determined that the k_{off} of the TD4-HlyA fusion is very low and could not be calculated (Figure 20). Furthermore, the K_D was calculated to be under 4 nM, since the lower concentration of TD4 did not reach the steady state with the binding times tested. This value is very similar to the apparent K_D of the V_{HH} domain alone (described by our group)[308], which means that the fusion of the V_{HH}s to the HlyA signal does not reduce the binding capabilities of the antibody. This affinity showed to be ten times higher than the observed affinity of Int280 (K_D = 40 nM) (Figure 19), the natural binder of TirM, which could be calculated both using the steady state approximation and relating the kinetic constants (k_{on} and k_{off}),

obtaining similar results. The k_{off} of Int280 could also be calculated to be $3.75 \cdot 10^{-2}$ s^{-1}, which is in any case higher than the one of TD4. While TD4 remains bound to TirM, the binding of Int280 is more likely to have less stability. These data are in concordance with previous observations indicating two-dimensional actin-based motility of EPEC on the cell surface[194], what suggests that the actin pedestals are dinamic. In fact, we have also observed (Figure 47, Video 1) that the pedestals formed by SIEC change with the time and bacteria still move along the cell surface even after the intimate attachment. Taking this altogether, the binding of TD4 could not only prevent the binding of Int at the infection site, but also displace it from the binding with Tir, while TD4 remains bound. During the infection the binding of Int280 gets tighter with the clustering of Tir on the cell surface, so the action of TD4 may loosen the intimate contact with the host cells by preventing the aggregation of Tir.

Identification of the site of attachment of TD4 to TirM

SPR experiments determined that the binding site of TD4 to Tir must be similar to the described binding region of Int280, as Int280 could bind TirM at a lower rate after the incubation with TD4 (Figure 21). To enclose the recognition site of TD4, we synthesized 12-mer overlapping peptides covering the whole sequence of TirM of EHEC on a PVDF membrane. We identified that TD4 bound to two consecutive non-overlapping peptides of the TirM sequence: VNIDELGNAIPS (296 - 307) and GVLKDDVVANIE (308 - 319) (Figure 22). V_{HH}s have longer CDRs that can adopt novel conformations[249] and recognise otherwise hidden clefts of the antigens, if they are compared with conventional antibodies. It can be inferred that one of the CDRs of TD4 may bind to one of the identified sites of TirM, while the other CDRs recognise the other peptide. But a more plausible explanation is that TD4 is recognising a 3-dimensional structure of the folded TirM and the CDRs still bind to the primary structure of the polipeptide, but with lower affinity. Other V_{HH} recognising the active site of lysozyme has been previously identified to bind rather more a terciary structure than a linear sequence of the target protein as a cognate antigen[51].

Furthermore, we determined that TD4 does not recognise the TirM homolog fragment of EPEC and only has a weak interaction with the CR homolog (Figure 24). This information led us to narrow the interaction site of TD4 and TirM of EHEC. We compared the TirM sequences of the three pathogens to identify amino acid changes in the peptides recognised by TD4. We consider that the differences between the proteins of EHEC and CR, which correspond with amino acids V309, N317 and E319, reduce but not abolish the interaction, as TD4 showed weak binding against the CR protein. On the other hand, the amino acid changes with the sequence of EPEC - amino acids E300, L301, V314 and A316 - are considered critical for the binding. Based on the crystal structure of the interaction interface between Int and Tir[214] of EPEC (Figure 23), we could enclose the amino acids that may participate in the interaction. Inside the TirM sequence, it has been identified an Int binding domain (IBD)[72] composed of two long alpha-helices (HA, residues 271-288, and HB,

residues 312-331) separated by a ß-hairpin (residues 294-308). The described structure reveals that the binding of Int to Tir is primarily mediated by interactions between the lectin-like D3 domain of Int and the ß-hairpin and the N terminus of helix HB of Tir IBD of EPEC, covering the corresponding residues 294-313 of TirM of EHEC. The identified peptides (296 - 319) are framed within the IBD sequence, indicating that TD4 is directly interfering with the Int:Tir interaction. Interestingly, some of the identified amino acids that are essential for the binding (E300, L301) or increase the affinity (V309) of TD4 towards TirM are localised within the Int:Tir interaction sequence. Interestingly, it has been determined that the corresponding amino acids N397, I398 and V309 in EPEC are key determinants of the affinity of Int for TirM[299]. Their proximity to the amino acids to which TD4 may bind indicate that TD4 may be blocking these binding sites. The other identified amino acids are localised outside of the Int:Tir binding sequence and might not be interfering with the binding of intimin to TirM. This might explain the SPR results that showed partial binding of Int280 even if TD4 was previously bound (Figure 21). The obtained data suggest the purification of the TD4:TirM complex to stablish if the identified amino acids are participating in the interaction. Additionally, mutagenesis of the amino acids would confirm their relevance for the interaction.

Generation of a model bacteria to study the intimate attachment of EHEC in mice

The results of new strategies that are being developed to combat the infection of EHEC alternatively to antibiotics have to be interpreted with caution, since they report EHEC infections performed in mice - with or without previous treatment with antibiotics to deplete the intestinal microbiota. The usefulness of this model is questionable, because it does not mimic the human infection in some aspects: EHEC does not form A/E lesions on mouse intestinal epithelial cells, nor do mice develop HUS[244]. A variety of other animal species, including gnotobiotic piglets[77], baboons[328], ferrets[393], and calves[76], have been used as models to study the virulence of EHEC. EHEC is able to cause AE lesions in suckling neonatal piglets and neonatal calves, but these animals are expensive and difficult to manage. In the infant rabbit animal model[263], EHEC colonizes the large intestine, forming A/E lesions, and causing diarrhea. However, the newborn rabbits lack the normal microflora that is present in an adult animal. The restricted mouse pathogen CR is, in contrast a well accepted model to test possible treatments against EHEC. As an example, it has been modified to express the Stx of EHEC for their study in mice[222].

The results obtained in this work encourage the delivery of TD4 in an *in vivo* model of the infection as a next step towards combating the infection of EHEC. But given that TD4 was not capable of inhibiting the Int:Tir interaction of CR, we first modified CR-TccP to generate a deletion in the LEE5 operon of the LEE PAI - thus mutating intimin, Tir and the Tir chaperon CesT - resulting in a strain (CR-TccPΔLEE5) that is unable to form actin pedestals on the infected cells (Figure 25). At the same genomic integration site, the LEE5 operon of EHEC was recombined, but conserving the natural regulation of CR, the promoter and the

termination sequence of the LEE5. We used a strain that already expresses the effector TccP from the genome[119], a coupling protein for the Tir-signalling pathway needed to assemble actin pedestals in EHEC[114] that is absent in CR. Unlike CR-TccPΔLEE5, CR-EHEC was able to rearrange the actin cytoskeleton beneath the bacteria (Figure 25). This observation reaffirms that heterologous effectors of different pathogens can be secreted through the different T3SSs[8] and that Tir and Int are functionally interchangeable among these A/E pathogens. To confirm that the strain was still capable of colonizing the mouse intestine we performed *in vivo* experiments using mice infected with CR-EHEC, in collaboration with the laboratory of Prof. Gad Frankel. The mice showed intestinal hyperplasia and colonization in the intestine (Figure 27), opening the possibility to use this modified strain to replicate the infection of EPEC or EHEC in mice, thus generating a more reliable mouse model for the study of the infection. CR-TccPΔLEE5 could be used as a negative colonization control, since it has been shown that Tir is esential for the colonization, pedestal formation and colonic hyperplasia of CR in mice[79].

With the intention of performing *in vivo* experiments in mice infected with CR-EHEC, we demonstrated that TD4 is capable of inhibiting the formation of pedestals on HeLa cells infected with this strain (Figure 26). This promising result encouraged the design of a delivery method of this V_{HH} to the mouse intestine. An interesting approach is the generation of commensal or probiotic strains stably expressing the HlyA secretion system, which could colonize the mouse intestine and prevent the infection of EHEC by the inducible secretion of TD4. Using the same strategy as for the modification of CR, we integrated the necessary genes of the HlyA system into the chromosome of a K-12 commensal strain (MG1655) and demonstrated that the resulting strain (HS-TD4) is capable of secreting the TD4-HlyA fusion to the culture medium (Figure 28). The probiotic EcN, which has been recently demonstrated to compete with EHEC for the colonisation of the mouse intestine[223], would be a very valuable alternative strain for a possible treatment to combat EHEC, as it harbours natural probiotic genes, such as iron uptake systems or microcins[333], that the commensal strains lacks. However, HS-TD4 would be a better model to assess the specific effect that TD4 makes in the interference of the infection of EHEC in mice. In a collaboration with the laboratory of Prf. Gad Frankel (Imperial College, London, UK) we will develop *in vivo* infection experiments using the strain HS-TD4, to test the effect of the secretion of TD4 in the mouse intestine. Recently, an engineered *E. coli* strain with a modified LPS mimicking the Gb3 receptor has been shown to protect mice against an otherwise fatal dose of Stx-producing *E. coli* strains[270], so a combination of the two strategies could enhance the effect of TD4.

However, other delivery strategies can be considered. The Gram-positive *Lactobacillus* strains can be modified to secrete beneficial molecules such as cytokines or enzymes against infectious and inflammatory diseases[67,339,381]. *Lactobacillus* has also been demonstrated to secrete antibodies for live immunotherapy. Modified lactobacilli to secrete recombinant antibodies against *Streptococcus mutans* reduced dental caries

in rats[188]. Other studies show that specific V_{HH}s prevent the adsorption of bacteriophages to the host bacteria[146] and reduce the severity and duration of rotavirus-induced diarrhoea in mice[266]. As an additional alternative, TD4 could be administered to mice using a passive immunisation strategy[330]. The V_{HH}s can be overproduced in bacteria or in yeast to obtain highly concentrated purified antibodies[249]. Interestingly, selected V_{HH}s recognising Stx toxins were administered in combination with whole antibody molecules for the treatment of HUS[359]. However, the use of purified antibodies is a costly strategy to develop a treatment. To circumvent this problem, some studies describe the production of antibodies in plants to be comparatively lower. The production of antibodies in edible tissues allows oral passive immunization at the gastric mucosal surface, but the antibodies can also be stored in seeds[373]. Antibodies contained in seeds enable long-term storage and the direct use for passive immunization with oral administration, which is particularly advantageous. In a recent study, V_{HH}s fused to the constant region (Fc) of immunoglobulins were stored in *Arabidopsis thaliana* seeds. Piglets fed with these seeds were protected against enterotoxigenic *E. coli* (ETEC)[372].

Injection of V_{HH}s recognising CapG into eukaryotic cells

The use of live bacteria has a great potential to deliver therapeutic proteins *in vivo*, in specific organs or tissues, where they can produce a continuous supply of the polypeptide. Bacterial properties of colonizing the gastrointestinal tract, target tumors and secrete or expose molecules on their surface are adapted to the development of bacterial.derived therapies. Therefore, an attractive possibility is to engineer bacterial strains to carry a functional T3SS able to deliver therapeutic agents intracellularly. We have previously demonstrated that an attenuated strain of EPEC (EPECquad) can be used biotechnologically to inject heterelogous model V_{HH}s into the eukaryotic cytoplasm of HeLa cells through the T3SS by fusing the V_{HH}s to the 20 amino acid N-terminal secretion signal of the EPEC effector EspF[29]. Here, we first demonstrated that two V_{HH}s recognising CapG - a protein that modulates the length of the actin filaments by capping the barbed ends in a PIP2-dependent manner[389] - can be secreted through the T3SS to the culture medium using EPECquad (Figure 29). We then showed that these V_{HH}s can be injected to the cytoplasm of MDA-MB-231 breast cancer cells with this strain by using fluorescence determination (Figure 30)[52]. This indicates that V_{HH}s can be injected into cell types other than HeLa with this strategy and that a wider repertoire of sequences is subject for the proper injection to the cytoplasm through the T3SS. Additionally, VcapG4 produced a phenotypic effect upon its injection to HeLa cells, indicating that it is functional inside the host cells. When VcapG4 was injected, the pedestals formed by EPEC had an unusual elongated phenotype (Figure 31) that may be due to a direct VcapG4-mediated effect on CapG activity, though VcapG4 shows no effect on the F-actin binding activity of CapG *in vitro*[367]. Alternatively, VcapG4 may affect to the interaction of CapG with other cytoskeletal proteins recruited to the actin pedestal, alone or in combination with the EPEC effectors. High affinity VcapG4 injected locally by EPEC at the site of pedestal formation could result in

the recruitment of more CapG and associated proteins to the pedestal site, triggering formation of new actin filaments in cellular projections. VcapG4 may alternatively alter other actin structures of the cell proximal to the EPEC pedestal site (e.g. focal adhesions), which could induce a partial retraction of the plasma cell membrane near the pedestal. Regardless of the mechanism by which VcapG4 acts on the EPEC actin pedestals, this data demonstrate that intracellular protein targeting by *E. coli* injection of V_{HH}s can modify a biological function of the cell.

Design of a Synthetic Injector *E. coli* (SIEC) strain

The use of extracellular bacteria harbouring a T3SS as active immunotherapy delivery systems for the injection of heterelogous proteins lack the need of transfering protein-encoding genes by viral infection or transfection[179]. This strategy has also been assesed using other microorganisms than EPEC. Effector-less[157], attenuated[94] or "killed but metabolically active" *Pseudomonas aeruginosa* that cannot replicate[196] and attenuated *Salmonella* strains[267,303,360,409] have been engineered for the delivery of heterelogous proteins. However, their use for the injection of proteins implies that even the attenuated pathogen could inject known or possible cryptic effectors to the target cells with the risk of affecting the host cells and even masking the effect caused by the injected proteins. To circumvent this problem, strategies based on commensal or probiotic strains are desireable. Functional studies have demonstrated that all of the necessary genes for the assembly of the T3SS on A/E pathogens are present in the LEE. In a previous approach, the complete LEE PAI of EPEC was transferred to *E. coli* K-12, but resulted in poor secretion of the trasnlocators[41,234]. Additionally, plasmids transformed into this strain totally abolished the expression of the T3SS (Ana Blanco, PhD thesis). In other study, an effector-less PAI of *Vibrio parahaemolyticus* has also been transferred to an *E. coli* K-12 strain, but the expression affected to the membrane integrity of the cells and the proteins detected in the supernatant were due to a leakage of the cytosolic proteins[2]. Besides, all of the approaches described above are subject to the complex internal regulation of the T3SS present in the PAI[25,156,195] and thus cannot be properly controlled.

To circumvent these problems, we assessed the possibility to engineer a *E. coli* K-12 strain expressing functional injectisomes by a "synthetic approach", in which we intentionally redesigned the chromosome of *E. coli* K-12 to integrate novel engineered LEE operons (eLEE) (Figure 32) under the inducible Ptac promoter. The projected strain was designed to lack all of the LEE-encoded or Nle effectors and the natural occuring regulators of the expression of the EPEC T3SS. Additionally, our aim was to reach a strain that would have less adhesion capacities than the commensal K-12 strain to avoid unspecific binding of the bacteria to eukaryotic cells. To this end, we selected loci that correspond with putative adhesins or cryptic adhesins (only expressed under specific circumstances) as integration sites of the chromosome[180,186,300]. These adhesins have been shown to be expressed under certain environmental situations and could

contribute to *E. coli*'s ability to adhere to and colonize a wide diversity of surfaces in its various ecological niches … . Additional genomic sites encoding other adhesins could serve for the integration of other genes of interest for future applications or we could just remove them to reduce the unspecific binding of the strain. For the modification of the genome of *E. coli* we used a strategy that leaves no antibiotic resistance in the site of integration[281], and allows multiple deletions or substitution of genes. First, we integrated GFP^{TCD} in the selected sites and observed that the fluorescent protein was expressed from all of the independent loci (Figure 33), thus indicating that there is no inhibitory effects derived from the overexpression of proteins in these genomic sites. However, the expression detected from some loci was differential, possibly due to epigenetic effects. In the case of *yeeJ*, we observed higher expression of GFP^{TCD}, what could be explained due to the presence of a tRNA gene upstream of the promoter, which is usually expressed constitutively in the genome.

We generated five eLEE operons (eLEE1-4 and eEscD) (Figure 32), all controlled under Ptac promoters and the natural terminators of the deleted adhesins. The transcriptional units encode all known structural components and chaperones needed for assembly of injectisomes, plus the translocators EspABD, but lacking the LEE-encoded effectors and the endogenous regulation found in EPEC (i.e, promoters, transcriptional regulators, terminators). The Ptac promoter allows a controlled expression of the genes to avoid possible toxicity derived from the expression of the T3SS genes and a controlled timing of the injection with the addition of the IPTG inducer. To select the pairing sites for the integration of the synthetic operons according to the GFP^{TCD} signals observed, we followed previous studies on the transcriptional activity of each of the operons. It has been described that the expression derived from the activation of the natural promoter of the LEE2 operon is stronger than the one of the LEE3 operon[40] and real time PCR studies have determined that the level of transcription of LEE4 is higher than the levels of LEE3 and LEE1 in EPEC, in that order[201]. According to this, we matched the eLEE2 and eLEE4 operons with the sites where GFP^{TCD} showed higher expression, while eLEE1 was associated with the site with less expression. We sequentially integrated these five eLEE operons in the selected sites of the chromosome of the *E. coli* K-12 Δ*fimA-H* strain (EcM1) obtaining SIEC, which lacks the following adhesion-related genes: *yfaL* (eLEE1), *yeeJ* (eLEE2), *yra* (eLEE3), *yebT* (eLEE4), *yfc* (eEscD).

Assembly of T3S injectisome on SIEC and its connection with the flagellum

SIEC was shown to secrete translocators EspABD to the culture medium (Figure 35), a characteristic that measures the activity of the T3SS[170] and implies that SIEC assembles functional injectisomes on its membrane. The presence of the translocators in the culture supernatant is due to the activity of the T3SS, since EcM expressing the eLEE4 (which harbours the EspADB proteins) alone did not exhibit secretion of these proteins in the culture supernatant (data not shown). Additionally, the assembly of the T3SS did not

show lysis or hindered the growth of the bacteria (Figure 34). The activity of the T3SS was compared with strain SIECΔp1, a mutant of SIEC that lacks the promoter of eLEE1 and showed no translocated proteins in the medium (Figure 36A). This indicates that the functional expression of the T3SS in SIEC depends on the correct expression of all of the operons. We also demonstrated the positive expression of proteins encoded in the different engineered operons (Figure 36B) and, by comparing the relative expression of the operons with the natural expression of EPEC, we observed a similar expression of the genes in SIEC. Interestingly, the expression level of EscC and EscJ, both in the eLEE2, was slightly higher relative to EPEC, as happened with the eLEE4 and eEscD, while the expression of EscN in the eLEE3 was not. Placing a stronger promoter to regulate the expression of eLEE3 might then enhance the overall T3-derived secretion. A more recent study[398] has determined that the transcriptional levels of LEE4 in EPEC are higher than the ones of LEE2, while we selected the site with the highest expression levels of GFP^{TCD} (*yeeJ*) to be the integration site for eLEE2. In future modifications of the strain, we could adapt the differential transcription levels of the eLEEs of SIEC to the natural ones, what may enhance the overall activity of the T3SS in SIEC, either by changing the promoter to stronger or weaker ones[33] depending on the operon, or changing the integration sites. Besides, the lytic transglycosilase (EtgA) expressed from the LEE has been demonstrated to enhance the assembly of T3S injectisomes in EPEC[112], though its overexpression caused cell lysis. A possible strategy to enhance the assembly of injectisomes could be the integration of this gene in SIEC under the regulation of a weaker promoter or in a genomic site where the expression is limited (e.g. the *sfm* locus).

Derived from the expression of the T3SS in SIEC, we observed that the secretion of flagellin (FliC) was reduced (Figure 36A), indicating that the expression of the T3SS genes affected to the assembly of the flagellum. Motility studies determined that the proteins expressed from the eLEE1 operon were responsible for this effect (Figure 39). The structural similarities and the common evolution[42,95] and even regulation[153] between the T3SS and the flagellum may explain this effect, since proteins of the eLEE1 have homologs of the flagellum. The T3SS components present in the eLEE1 are EscE, CesAB, EscL, EscR, EscS, EscT, EscU and a protein with unknown function (*orf4*). One or some of these components may be interacting with the flagellar assembly machinery thus inhibiting the assembly of flagellin. EscRST are homologs of FliPQR, integral membrane proteins that are essential for the flagellar function and are believed to assemble within the IM ring at the cytoplasmic interface of the basal body. EscL is the regulator of the EscN ATPase and is homolog to FliH. FliH also regulates the ATP-hydrolyzing activity of the flagellar ATPase and is essential for the export of the subunits that assemble the rod, the hook and the filament of the flagellum[242]. We believe that one or a combination of these flagellar homolog proteins encoded in the eLEE1 operon might be responsible for the interference with the flagellar assembly. Interestingly, this inhibition was not bidirectional, as the mutation in the master regulators of the flagellum (*flhDC*)[204] did not result in augmented secretion of the T3S translocators (Figure 39). Interestingly, EcM1 is derived from MG1655 strain, which harbours an insertion element (IS1) upstream of the master regulators of the

flagellum[204] that conferres hypermotility[115], so the interference of the expression of the T3SS just partially reduced the motility.

When observed at the electron microscope, induced SIEC harbours filaments on its membrane (Figure 40) that are typical for the T3SS expressing bacteria[69]. Additionally, the needle complexes of SIEC were isolated as described before[325] in paralell with the EPEC injectisomes to observe the macromolecular structure of the T3SS at the electron microscope (Figure 42). The identified injectisomes showed to be highly similar to the ones of EPEC, thus indicating that the engineered expression of the T3SS components of EPEC can result in the assembly of the same macromolecular complexes that are isolated from the pathogen. SIEC was also demonstrated to be able to form translocation pores[245] on the membrane of erythrocytes (Figure 43), thus indicating that EspB and EspD are secreted to the supernatant in a functional form. Additionally, SIEC could inject Tir, a model natural effector of EPEC, into the cytoplasm of host cells (Figure 45). We selected Tir because of the perceptible phenotypic effects that causes its translocation to the host cells: the intimate attachment of the infecting bacteria and the formation of actin pedestals at the site of the infection[177]. SIEC showed all of these attributes, thus indicating that Tir was injected to the host cells, that it was functional and that we can mimic the natural phenotype of the pathogenic EPEC *in vitro* using a genetically modified strain derived from a commensal *E. coli*.

Very interestingly, longer infection times of SIEC to HeLa cells showed that the bound bacteria were internalised (Figure 46, Video 1). This process is not observed in EPEC, as it remains an extracellular pathogen. We hypothesize that this process may be happening in SIEC due to the unregulated injection of Tir compared to EPEC[24] and/or the lack of certain factors that EPEC may express or inject to remain extracellular. Though this observation needs further investigation, we propose EspB to be responsible of this effect. A region of EspB (amino acids 159-218) binds to α-catenin, α1-antitrypsin and to the actin-binding domain of several myosin proteins[152]. By inhibiting interactions between myosins and actins, EspB can prevent both the initiation of phagocytosis and the effacement of microvilli upon the infection of EPEC. It has been reported that EPEC harbouring a deletion of this region of EspB is still able to translocate virulence factors to host cells[132]. Thus, a possible explanation of the internalisation is that EspB is not injected in enough amounts to prevent phagocytosis.

Injection of heterologous molecules and proteins using SIEC

Taking the results altogether, the recombinant SIEC strain could serve as a vehicle for the administration of heterologous proteins into the cytoplasm of target cells. The previous approaches using attenuated or "non-living" T3SS-expressing pathogens[157,196] do not ensure that bacteria may not be delivering possible cryptic effectors to the eukaryotic cells. SIEC is, in contrast, a commensal *E. coli* strain that resides

harmlessly among the intestinal microbiota. Additionally, the injection done by the T3SS of SIEC follows a synthetic approach. The expression of the T3SS is regulated by heterelogous promoters that could allow us to monitor the injection of proteins once the bacteria are attached to the cells. In fact, SIEC had much higher secretion yields than a commensal strain harbouring the complete LEE PAI (Figure 37). In this work, we have used a model effector of EPEC for the injection, but other effectors or heterologous proteins can be subject for their injection with SIEC. As possible effectors of EPEC or other A/E pathogens to be injected, those encoded in the LEE PAI - specially EspG, EspH, EspF, map and EspF - are related to cytotoxicity and apoptosis[73], so they are good candidates for their injection to, for example, tumor cells. In the case of the Nle effectors, most of them function as supressors of the immune cell response and could be used for the treatment of the inflammed tissue in patients with Crohn's disease or intestinal bowel disease so to reduce the autoimmune response in the intestine.

Another interesting application would be to deliver therapeutic V_{HH}s intracellularly for the targeting of proteins involved in diseases such as inflammation and cancer in the gastrointestinal and even in the urinary tract. Importantly, V_{HH}s interfering with the function of relevant intracellular targets have already been selected. A V_{HH} sequence has been shown to activate caspase-3 and induce apoptosis[235], while a VH antibody has been described to bind activated GTP-bound RAS and prevent cell proliferation[345]. Transfected V_{HH} sequences against gelsolin were able to block the interaction with G-actin delaying cell migration[365], a similar effect shown by one of the VcapG V_{HH}s[367].

We could even take advantage of bacterial toxins for their injection to human tumors. The therapeutic potential of Stx has been suggested, given the high expression of Gb3 in some cancers, which could facilitate a selective release of A fragment only in tumor cells[93]. In the absence of the pentameric B subunit, the catalytically active A fragment is not internalized by cells neither can access the cytosol, so the injection with SIEC would be a safe delivery method. SIEC could also be equipped with genes encoding other bacterial toxins, such as cytolysin A[158] or genes encoding for pro-drug converting enzymes that locally transform non-toxic pro-drugs into cytotoxic drugs[198]. As examples, the herpes simplex virus thymidine kinase (HSV-TK) or the *E. coli* cytosine deaminase (CD), convert the pro-drugs ganciclovir and 5-Fluorocytosine into ganciclovir monophosphate and 5-Fluorouracil, respectively, but only in those cells that express the enzymes. Additionally, bacteria have also been engineered to induce apoptosis in tumor cells, by secreting tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)[111] and Fas ligand[208], or inducing an immune response against tumor cells by secreting specific cytokines[207].

Other interesting molecules include transcription factors, which could be used to efficiently modulate the temporal activation of gene expression and direct the cell fate without risking the genomic integrity, as an alternative to transgene expression, that can cause potential insertional mutagenesis[157]. The muscle

regulatory factor MyoD has already been used for its injection in fibroblasts using attenuated *Pseudomonas*. As a result, the cells were differentiated into functional myocytes[26]. In the same direction, Transcription Activator-Like Effector Nucleases (TALENs) that recognize specific DNA sequences have been used to artificially manipulate gene expression and introduce double-strand breaks, triggering non-homologous end joining and homologous recombination[157].

To ensure the delivery of the therapeutic molecules at the right moment, we can engineer the expression of the T3SS injectisomes and the therapeutic proteins to be under the control of alternative inducible promoters that, unlike the Ptac, have stable non-toxic inducers appropriate for their use *in vivo*, such as the tetracycline promoter (Ptet) or the arabinose promoter (PBAD). The inducers of these promoters can be administered orally and intraperitoneally to induce *E. coli* when colonizing solid tumors[209]. In addition, these are strong *E. coli* promoters that are tightly regulated[124,130,331], ensuring a strict control of the expression of the T3SS injectisomes and of the therapeutic protein to be injected. Furthermore, the targeting of SIEC to specific cells can be implemented based of the expression of Synthetic Adhesins[279], which are expressed on the bacterial surface and recognise specific antigens on the surface of tumors. For assays requiring prolonged incubation of the cell cultures, bacteria may be killed with antibiotics (e.g. ampicillin, gentamicin) after a defined time of injection. In conclusion, the SIEC strain can be considered as a chassis to be modified for the directed injection of a wide variety of molecules to specific cell types and tissues.

CONCLUSIONS

1. We have generated an immune library of V_{HH}s against EHEC protein EspA, and protein domains Int280 and TirM, essential for the Int:Tir interaction and the formation of A/E lesions. We used phage display selection to enrich positive binders against these antigens and employed the HlyA-secretion system to make the final screening for specific and high affinity clones against each of these proteins from the supernatants of the producing *E. coli* cultures.

2. High affinity V_{HH} clones against EHEC EspA, Int280 and TirM were purified and characterized *in vitro*. V_{HH}s against Int280 (IB10) and TirM (TD4) blocked Int280-TirM interaction *in vitro*, while only TD4 showed inhibition of actin pedestal formation during the infection of EHEC on HeLa cell cultures. The presence of TD4 during the infection reduced the number of EHEC bacteria intimately attached to HeLa cells.

3. We determined by SPR that the affinity of TD4 to TirM (K_D = 4 nM) was ten fold higher than that of Int280 (K_D = 40 nM). TD4 did not show significant dissociation from TirM upon binding whereas Int280 was dissociated with a k_{off} of $3.75 \cdot 10^{-2}$ s^{-1}. SPR data also showed that Int280 could still bind TirM in the presence of TD4, albeit with a reduced level, indicating that TirM has overlapping but not identical binding sites for TD4 and Int280. TD4 bound to the TirM peptides VNIDELGNAIPS and GVLKDDVVANIE, which are only partially conserved in Tir proteins from EPEC and CR. TD4 did not bind EPEC TirM and showed low affinity for CR TirM.

4. We engineered CR to express Int, Tir, CesT and TccP proteins of EHEC generating Citro-EHEC strain, which was able to form actin pedestals on HeLa cells and to colonize the mouse intestine inducing crypt hyperplasia. This leaves open the possibility to use this strain as a mouse model for the formation of EHEC A/E lesions. Purified TD4 inhibited the formation of actin pedestals upon infection of Citro-EHEC to HeLa cells.

5. We have generated an engineered *E. coli* K-12 strain, named Synthetic Injector *E. coli* (SIEC), which assembles functional T3SS injectisomes of EPEC upon induction with IPTG. The genes encoding EPEC injectisomes were organised in four engineered operons (eLEE1-eLEE4) lacking effectors and transcriptional regulators and one monocistronic transcriptional unit (eEscD), all under the control of the inducible *E. coli* promoter Ptac. These genetic construct were integrated in the chromosome of *E. coli* K-12 at specific sites that correspond with to genes and operons encoding fimbrial and afimbrial adhesins to reduce the non-specific adhesion of SIEC to mammalian cells.

6. T3SS translocators EspA, EspB and EspD were secreted in the medium of induced SIEC cultures grown in LB. Injectisomes with EspA filaments were detected by transmission electron microscopy on the surface of SIEC bacteria and were purified from the cell envelope as needle-like filamentous structures alike those

found in EPEC. The induced SIEC strain also showed haemolytic activity on erythrocytes indicating the functionality of EspBD translocation pores.

7. We have demonstrated the protein translocation capacity of SIEC injectisomes on HeLa cells. SIEC expressing Int, Tir, and CesT of EPEC (eLEE5) was shown to inject Tir into HeLa cells, which was inserted in the plasma cell membrane recognising Intimin on the bacterial surface and polymerising actin pedestals underneath the attached bacterium.

1. Hemos generado una genoteca inmune de V_{HH}s frente a las proteínas de EHEC EspA, Int280 y TirM, que son los dominios esenciales para la interacción Int:Tir y para la formación de las lesiones A/E. Usamos la técnica de selección "phage display" para enriquecer la genoteca en clones positivos para la unión a estos antígenos. Finalmente empleamos el sistema de secreción de HlyA para secretar los V_{HH} al sobrenadante de cultivos de *E. coli* inducidos y hacer un rastreo final de clones específicos y de alta afinidad.

2. Purificamos y caracterizamos *in vitro* los V_{HH}s de alta afinidad frente a EspA, Int280 y TirM de EHEC. Identificamos clones frente a Int280 (IB10) y TirM (TD4) que bloqueaban la interacción Int280-TirM *in vitro*, aunque sólo TD4 mostró actividad inhibitoria de la formación de pedestales de actina durante la infección de EHEC a células HeLa en cultivo. Además, demostramos que la presencia de TD4 durante la infección reduce el número de bacterias unidas íntimamente a las células HeLa.

3. Determinamos mediante SPR que la afinidad de TD4 por TirM (K_D = 4 nM) era diez veces mayor que la de Int280 (K_D = 40 nM). TD4 no mostró una disociación significativa de TirM, mientras que Int280 se disocia con una k_{off} de $3.75 \cdot 10^{-2}$ s^{-1}. Los datos de SPR también mostraron que Int280 puede unirse a TirM aún en presencia de TD4, pero a niveles menores, lo que sugiere que los sitios de unión a TirM de estas proteínas se solapan pero no son idénticos. TD4 se unió a los péptidos VNIDELGNAIPS y GVLKDDVVANIE de TirM, los cuales están conservados sólo parcialmente en EPEC y CR. TD4 no se une a TirM de EPEC y muestra baja afinidad de unión a TirM de CR.

4. Hemos modificado CR para que exprese las proteínas Int, Tir, CesT y TccP de EHEC generando de esta forma la cepa Citro-EHEC. Esta cepa es capaz de formar pedestales de actina sobre células HeLa y de colonizar el intestino de ratones produciendo hiperplasia de la criptas intestinales. Esto abre la posibilidad de establecer modelos murinos de formación de lesiones A/E de EHEC usando esta cepa. Además, hemos comprobado que el sdAb TD4 purificado inhibe la formación de pedestales de actina en cultivos de células HeLa infectados con Citro-EHEC.

5. Hemos generado una cepa modificada de *E. coli* K-12 que ensambla inyectisomas funcionales del T3SS de EPEC al inducir su expresión con IPTG, denominada *E. coli* inyectora sintética (SIEC). Los genes que codifican los componentes de los inyectisomas los organizamos en cuatro operones artificiales (eLEE1-eLEE4) que no contienen genes de efectores o reguladores transcripcionales y una unidad transcripcional monocistrónica (eEscD), todos bajo el control de promotor Ptac de *E. coli*, inducible con IPTG. Estas construcciones genéticas se integraron en el cromosoma de *E. coli* K-12 en sitios específicos que codifican adhesinas fimbriales y no fimbriales, con el fin de reducir la adhesión no específica de SIEC a células de mamífero.

6. Comprobamos que los translocadores EspA, EspB y EspD del T3SS se secretan al medio en cultivos de SIEC crecidos en LB e inducidos con IPTG. Los filamentos de EspA de los inyectisomas se detectaron por microscopía electrónica de transmisión en la superficie de SIEC y se purificaron de la membrana bacteriana. Se observaron estructuras filamentosas tipo aguja similares a las encontradas en EPEC. La cepa SIEC inducida también mostró capacidad hemolítica sobre eritrocitos, indicando que los poros de translocación formados por EspBD son funcionales.

7. Hemos demostrado que los inyectisomas de SIEC son capaces de translocar proteínas a células HeLa. Comprobamos que una cepa SIEC modificada para expresar Int, Tir y CesT de EPEC (eLEE5) inyecta Tir a células HeLa, que se insertó en la membrana plasmática de las células infectadas, reconoció Int en la membrana de la bacteria y polimerizó pedestales de actina en los sitios de unión de la bacteria a la célula infectada.

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